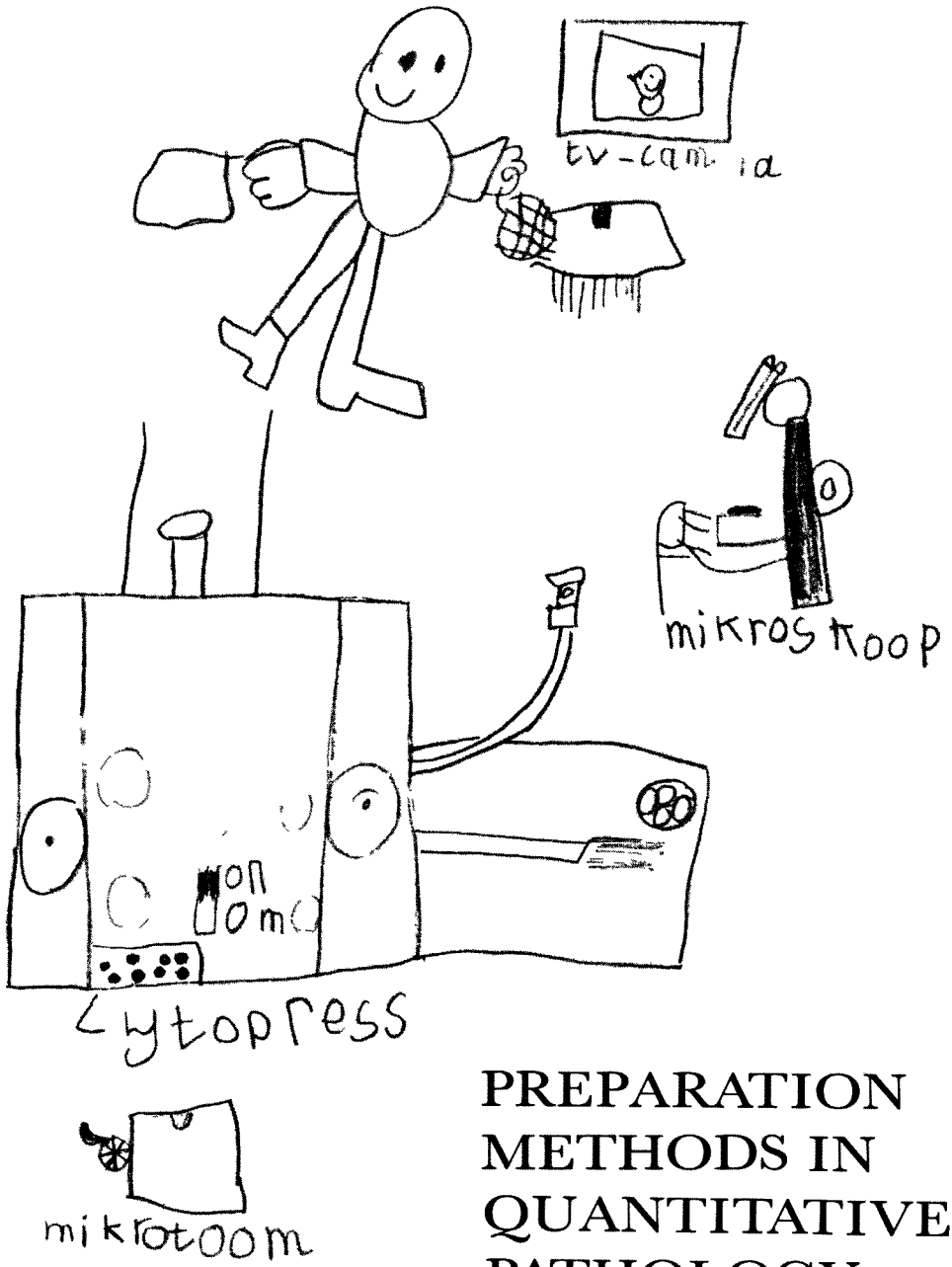

PREPARATION METHODS IN QUANTITATIVE PATHOLOGY

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN DE RECTOR MAGNIFICUS, PROF. DR. J H.G.I. GIESBERS, VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN IN HET OPENBAAR TE VERDEDIGEN OP DONDERDAG 20 FEBRUARI 1986 DES NAMIDDAGS TE 4.00 UUR

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Cover: "Papa's werk (My father's work)" by Matthijs and Ilse Oud (with some technical advice of Annemieke Oud).

VOORWOORD

Zoals zovele andere proefschriften is ook dit proefschrift niet het werk geweest van één persoon. Aan de tot standkoming hiervan hebben o.a. tweeëntwintig mede-auteurs en een meervoud aan andere medewerkers en afdelingen, zowel binnen de Katholieke Universiteit van Nijmegen als daarbuiten, hun medewerking verleent. Zonder eenieder nu bij naam te noemen (bepaalde personen mogen zelfs niet genoemd worden, anderen staan reeds bij de diverse publikaties vermeld) wil ik iedereen, die op haar of zijn wijze heeft bijgedragen tot het tot stand komen van dit proefschrift, bedanken. Ik hoop dat eenieder zijn bijdrage aan dit proefschrift hierin terug zal vinden.

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Voor Tineke, Matthijs, Ilse
en Annemieke
Aan mijn moeder
Aan mijn familie

TABLE OF CONTENTS

CHAPTER I

GENERAL INTRODUCTION

The diagnosis of malignant, borderline malignant, pre-malignant and normal cells and tissues by pathological anatomical techniques is based primarily on light microscopical evaluation of cytological and histological material. This diagnostic screening requires a high degree of skill and experience however, and interpretation is subjective.In many cases, the diagnosis made by the pathologist is reliable enough to provide a basis for clinical decision making. However, discriminating between pre-malignancy and malignancy and rendering a reproducible grading of pre-malignancies and malignancies remains a very difficult matter. Considerable inter- and intra-observer variations have been reported both for the interpretation of histological (4,21,27,28,33,36, 40,54,63,67) and cytological (7,23,65) material. In recognition of the imcompleteness of this descriptive morphological screening additional means to supplement this qualitative evaluation have been developed, such as enzyme staining and immunohistological techniques (60). A relatively recent and rapidly developing adjunct to standard anatomical pathology techniques is quantitative pathology, in which cytological and histological features are expressed numerically. At present, there are two general types of quantitative pathological methods available. These are image analysis-derived techniques (29,35,39) and flow cytometric techniques (8,14,25,34,35,39,44,47,64). Image analysis (or image processing) techniques are used in the quantification of morphological and histochemical parameters in microscopical preparations of cytological and histological material. The concept of flow cytometry is comparatively new (39). Parameters are measured in single cells, mostly stained with fluorescent dyes, in suspension. Therefore fluorescent signals but also light scatter, light absorption or electrical resistance of the cells, correlating with morphological and histochemical

parameters, are measured. Supplementing routine light microscopic examination with image analysis and flow cytometry can provide a marked improvement in reproducibility of tumor diagnosis and grading. In addition they may provide new quantitative insights into clinical aspects of tumor behaviour and development and thus form the basis for significant changes in routine screening and diagnosis.

A standardized and well-controlled specimen preparation procedure is a prerequisite to quantitative analysis in pathology. Specimen preparation can be subdivided into cell (tissue) processing and staining. The methods required for image analysis link up with conventional methods, in which cells and tissues are put and stained on glass slides. Essential to all flow cytometric investigation are monodispersion and staining of the cells in suspension, demanding completely different techniques from those used in routine cytology.

Preparation methods in image analysis

Image analysis measurements make use of techniques varying from simple manual counting methods to fully automated, computer-assisted procedures.

One technique familiar to pathologists involves the counting of mitotic figures within a microscopic field. This technique has been applied, for example, in the prediction of the malignant behaviour of breast cancer (6). Another simple form of quantitative assessment is point-counting, using grid patterns overlaid on photomicrographs (5,35) and counting "hits" over different components. This method is well-suited to obtain data for calculating volume density or surface area in complex preparations in which many components are mixed (2,3). This procedure is a form of morphometry, which is broadly defined as the measurement or estimation of distance, area or volume, deduced from two-dimensional structures as observed in a microscopic image. Planimetry (5,35) and

stereology (5,82,83) are related terms used to describe the analysis of images in either two or three dimensions, respectively. A simple unidimensional measurement procedure involves the use of a micrometer inserted in the eyepiece of the microscope (76). A significant limitation of this technique is however, that it restricts the ability to measure irregular two-dimensional areas. This can be overcome by using a drawing prism and planigraphy, but this is time consuming. Therefore a device called the digitizing pad (or graphic tablet) (5,35) has been constructed for assessing two-dimensional structures. Structures of interest in a projected microscopic field or photomicrograph are outlined on this tablet by a human operator. The coordinates are fed into a computer and morphological features are then automatically determined.

All of the techniques mentioned above are interactive. They use the human observer's capacity to eliminate artifacts, overlapping or damaged cells or nuclei and other unmeasurable elements and provide a data collecting system (e.g. a computer) directly with images suitable for analysis.

Technically more advanced forms of image analysis make use of so-called scanning systems (9,15,16,29,59, 66,68,90). Microscopic images are measured when slides on a microscope stage under computer control are moved under a photosensing device such as a photomultiplier tube, diode-array or television camera system. Morphometric and photometric information can be obtained simultaneously.

An important attribute of these image analysis systems is their overall spatial resolution. This is a function of the microscope optics and the sampling resolution of the photosensing device. High resolution measurements up to the level of the resolving power of the optics can be made. In this way in addition to for instance area measurements the delicate chromatin texture in the nucleus can be measured. Another important attribute of these systems involves the photometric properties which enable the quantification of histochemical parameters. In many

cases, there is a direct relationship between the amount of bound dye and the amount of macromolecules present in the cell. The amount of dye can then be determined by scanning and integrating the absorbance of the light in a certain area of the specimen. In such a way for instance it is possible to quantify total cell protein (71).

A first step in these fully automated measurements of objects by a scanning device is the recognition of the structures of interest and the elimination of artifacts. This differs significantly from the interactive methods of image analysis, where this was done by a human operator. It will also imply special demands on the specimen preparation procedure (see below).

A major prerequisite for all image analysis techniques (interactive and automated) is a standardized method of specimen preparation that will give reproducible measurements. Differences in the processing of the material may have considerable influence on the resulting measurements, as shown for instance with the analysis of nuclear features in urological cytological specimens that have been processed differently (12). For measurements in histological sections, standardization of fixation (75,79) and sectioning of the tissue (52) are extremely important.

Conventional histologic sections can be used for measuring such features as area, stroma/epithelium ratio etc. When parameters such as DNA or protein content per cell nucleus are to be measured, however, sections are unsuitable as the majority of nuclei are cut. Moreover, there is always the chance that nuclei in different planes of the section overlap. This necessitates the development of entirely new preparative techniques for well-spread cells and/or nuclei to enable automatic quantitative analysis, and the gathering of data on the high resolution level such as chromatin texture. A recently introduced nuclear preparation method from histological, paraffin embedded tissue (30,78) is a promising new technique since retrospective studies on clinically well-documented material are now made possible.

Most conventional cell processing procedures make use of manual methods to deposit cells onto glass slides, without a previous assessment of cell number in the sample. This results in preparations with varying cell densities and, moreover, irregular cell distributions. Overcrowded specimens are found as well as slides with very few cells present, both resulting in inadequate measurement results. These problems have led to the development of new processing techniques, which have been developed initially in the field of cervical cytology (22,55,81, chapter 2 of this thesis).

Apart from a standardized processing of the cells and tissues, a reproducible staining technique is also needed. When interactive forms of image analysis are used to determine only morphological features, no extreme demands have to be put on the staining procedure. The human operator has to recognize and indicate the structures to be measured. When fully automated measurements are made, and also histochemical parameters are to be obtained, standardization is of utmost importance. This will include the use of pure dyes and well-controlled staining times and conditions (e.g. pH, temperature). In diagnostic pathology two stains, the Romanowsky-Giemsa (10,45,48,49,86) and the Papanicolaou stain (26,50,87) have been standardized. A critical review concerning standardization of these stains has been published recently (88).

Conventional staining procedures, even when standardized, remain less suited for fully automated measuring. Most cells have widely different absorption characteristics, both from cell to cell, as well as within the same cell (e.g. between nucleus and cytoplasm). Measurements at different wavelengths (38,72), together with sophisticated and time-consuming computer programs (46,53,85) are then necessary to obtain the relevant parameters. Although most research uses conventional stains, new staining procedures have also been developed,

especially for automated cervical cytology (18,22,69, section 3.1 of this thesis) allowing one to determine different nuclear features at a single wavelength.

A comprehensive review of image analysis applications to tumor pathology has been published recently (29).

Preparation methods in flow cytometry

Flow cytometry, enabling the measurement of different cell parameters, e.g. DNA (19), enzyme activity (11), cytoskeletal proteins (24,61) etc., in suspension, has resulted in a fast (up to 10.000 cells per second) and sensitive (as few as 2000 dye molecules per cell) new methodology. However there are some drawbacks to this technique. The fact that single cells in suspension must be measured excludes from analysis tissues and tumors that are difficult to disaggregate. Furthermore the relationship between groups of cells, or from one cell to another cannot be reconstructed with analysis of cells suspended from a tissue. Finally it is generally difficult to visually inspect the specimens that are measured. This last disadvantage can be partly overcome by using flow cytometers with sorting capabilities (25,47,58,77). Cells of interest, meeting preset parameter values, can be sorted out for visual inspection (56), or for other studies, e.g. image analysis measurements (56,70, section 3.3 of this thesis).

The application of flow cytometry in tumor pathology has gained widespread acceptance particularly in the diagnosis of malignant lymphomas and leukaemias which is described in detail elsewhere (8,34,44,47,64).

As already stated monodispersion is an absolute requirement for flow cytometry. Different procedures have been described for the disaggregation of cytological (42,51,84,89) and histological (1,13,17) material. Since the preparation of intact single cells can be difficult because of damage to the cytoplasm, and since in many cases only nuclear parameters are measured, nuclear

isolation procedures are also used, both for unfixed (41,43,57,74,80) and fixed paraffin embedded tissue (30,31,32,62).

Staining procedures in flow cytometry almost exclusively use fluorescent dyes. Simple one step methods for staining DNA (19,73), up to multistep procedures necessary for the detection of a variety of parameters (20,24,61) have been described. An extensive review of the different applications of stains has been published elsewhere (64).

Aim of the present study

In this study cell processing techniques (chapter 2) and staining procedures (chapter 3) for image analysis and flow cytometric measurements in cytological and histological material, are developed and evaluated. Subsequently these techniques are applied to the measurement of normal and malignant endometrium (chapter 4).

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CHAPTER II
CELL PROCESSING

SECTION 2.1

The development of a cervical smear preparation procedure for the BioPEPR image analysis system

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The Development of a Cervical Smear Preparation Procedure for the BioPEPR Image Analysis System

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The preparation procedure for the BioPEPR automated image analysis system is described Cervical cells are collected in a preservative solution and disaggregated by an automated syringing apparatus, giving about 50% single cells After centrifugation, the preservative solution is discarded, 2% polyetheleneglycol in 50% ethanol (carbowax) is added, and the cell solution is automatically spread onto a glass slide, resulting in a cell density of about 50 cells/sq mm in the central area of the smear Increasing cell density is shown to lead to a gradual linear decrease in single cells

All preparation steps have been critically evaluated, and the possibility of implementing this preparation procedure in a fully automated system is discussed

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Much research has been done on the preparation of cervical samples suitable for measurement by an automated image analysis or flow system. For such systems, cervical scrapes are usually collected in a preservative solution This suspension contains single cells as well as sheets and clusters of cells. To disaggregate these sheets and clusters, one or more mechanical means with or without a combination of (bio)chemicals have been tried. The most successful method, first reported by Tucker and Gresham,[15] is syringing Good results have also been demonstrated using ultrasonic agitation [1] To supplement the mechanical methods, chemicals and enzymes have been used to break the bonds between the cells Although many techniques have been tried, very little success has been reported. Quite often a successful disaggregation is accompanied by severe cell loss and/or damage. Only the use of dithiothreitol,[4] a combination of ribonuclease, dithiothreitol, iodoacetic acid, EDTA and Varidase[6] and some complex media according to the prescription of Escobar and co-workers[2] have been reported to improve on the mechanical methods

The quality of the cervical sample can be improved further by eliminating artifacts, which can be present in the cervical sample at the outset or may be introduced by the collection and disaggregation methods. Such artifacts can be eliminated by simple filtering using a sufficiently small pore size.[16] Another method is specimen enrichment using a density column.[8 13] Care has to be taken with both methods that no essential information originally present in the sample is lost.

Image analysis systems require an additional important step to deposit the cells onto a slide. Several methods have been tried: centrifugation,[1 5 11 12], allowing the cells to settle onto a slide,[4] spinning the slide,[17] filtering the cells and then pressing the filter against a slide[14] and smearing the cells suspended in a

carbowax solution onto a slide.[10] To optimize the adhesive qualities, coating the slides with polylysine,[1,4,5] albumin[5,17] and silicone[5] has been tried.

The only system reported to date that does most of the above-mentioned handling semiautomatically is that described by Bahr and co-workers.[1] In their system, syringing is used to disaggregate the cells, a cell count is made by measuring light scatter and absorption, and a fixed number of cells is placed on the slide by centrifugation.

This paper describes the preparation procedure for the BioPEPR image analysis system.[18] This procedure was chosen after evaluation of many different preparation procedures. In addition, the possibility of implementing this procedure into a fully automated system is discussed. This is necessary for an automated image analysis system, like BioPEPR, that is to be used in mass screening. The procedure has to fulfill certain demands, many of which have already been outlined by other authors.[1,7,11,16] Some additional important points are: (1) the preparation and staining procedure must be faster than conventional methods using the same or a lower number of persons; (2) the procedure may not introduce new artifacts, or, if it does, they must be easily recognizable; (3) the recovery of cells at the end of the procedure must be high enough that a reliable cytologic diagnosis can be made by either a machine or a person; (4) the diagnosis may not be altered by the preparation procedure.

Materials and Methods

The following procedure is used for the routine preparation of cervical smears. Up to 120 samples per day can be handled by one person. Cervical scrapes are taken with a plastic Ayre-type spatula and rinsed into 9 ml of preservative solution in a container with a built-in point (Sterilin, England), chosen so it can also be used for centrifugation later. The plastic spatula has the advantage that the cells can be easily rinsed off; earlier experiences with wooden spatulas showed that in samples with much mucus the scraped material tended to stick to the spatula. The preservative buffered solution (PBS) contains 110mM NaCl, 27mM Na_2HPO_4-$2H_2O$, 6mM KH_2PO_4 and a certain percentage of ethanol. In experiments where the samples are processed a day after the scrape is taken, 10% ethanol is used; in other cases, 20%. The buffer concentration is about twice that used in normal PBS. This was done because the pH may change due to the acidity of the cervical scrape. Preliminary experiments have shown that the pH in normal PBS sometimes drops from 7.0 to 6.7; this variation may influence later staining. The samples are kept at 4 C and processed one to five days after collection, although they have been kept for at least 30 days without any severe morphologic damage.

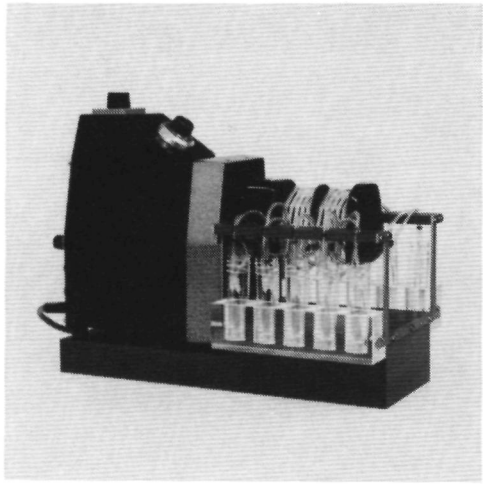

Figure 1
Peristaltic pump used for syringing the cell suspension.

The cells are disaggregated by syringing. To automate this process, a commercially available peristaltic pump (Watson-Marlow, England) is used (Figure 1). It is possible to syringe ten specimens at a time with this device: ten loops of tubing with 19-gauge needles at each end are connected to the pump. The solutions are pumped continuously through the tubes and needles, and the pumping direction is reversed several times in the beginning of the syringing procedure to clear the needles of possible large clumps. Syringing 8,000 samples has shown that hardly any blocking of the needles occurs. Experiments have shown that syringing for 15 minutes at a speed of 55 ml/minute gives optimal results. After syringing, the needles are discarded and the tubes rinsed with tap water and deionized water. Two of these devices are used simultaneously, allowing 20 specimens to be handled at a time.

Next, a 100μl sample is taken, diluted in 10 ml of Isoton (Baker's Diagnostics, U.S.A.) and counted by means of a Coulter Counter (Coulter Electronics, Hialeah, Florida). The counting thresholds are set in such a way that only cells larger than leukocytes are counted. The results of the counting are written on

the sample containers; these are then centrifuged for ten minutes at 4,000 rpm. The supernatant is discarded, and enough of a carbowax solution (2% polyethyleneglycol in 50% ethanol) is added to ensure an equal cell concentration in all samples. The proper amount of carbowax is dispensed by typing the result from the Coulter Counter into an automatic device (Figure 2). The apparatus has an accuracy of 5% to 15% for small volumes (<100μl) and about 3% for larger volumes. The cells are resuspended using a vortex mixer, and 35μl is spread on a glass slide by means of an automated smear preparation device used in hematology, the Hemaprep (Figure 3; Geometric Data, U.S.A.) This device draws the cell solution over the slide by means of another glass slide. The cells are air dried and then fixed. Depending on the staining, they are fixed in either a methanol-formaldehyde-acetic-acid-based mixture (for thionine-Feulgen-Congo-red staining[9]) or in 95% ethanol (for Papanicolaou staining).

An example of the cell distribution using this procedure is shown in Figure 4. By adjusting the cell concentration in solution, a mean cell density of 50 cells/sq mm was obtained in the central part of the smear. In practice, the cells remaining in the sample container are stored by adding an additional 2 ml of carbowax; cells can be kept in this solution for at least a year.

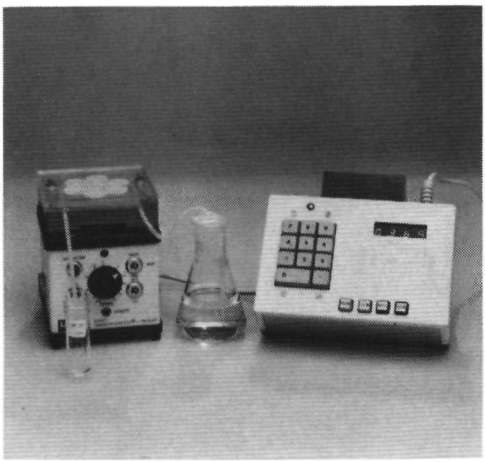

Figure 2
Automatic carbowax dispenser. Left to right: sample container, peristaltic pump, carbowax solution and electronics unit. The Coulter Counter result is typed into the electronics unit that drives the peristaltic pump to give the desired amount of carbowax.

Figure 3
Commercial blood smear apparatus used for spreading cervical cell suspension onto slides.

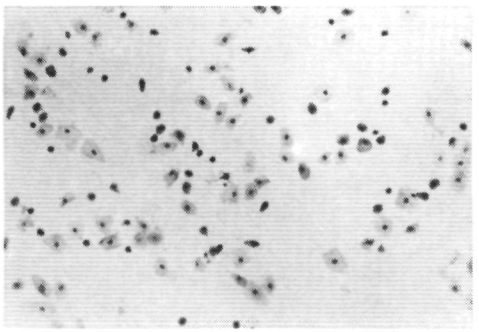

Figure 4
Cell dispersion in the central part of a cervical smear prepared according to the standard procedure (×125).

For the disaggregation experiments, cervical specimens were collected in the same preservative solution but without ethanol. After standing for one to three hours at 4 C, the specimens were divided into three or more parts. A cell count was done on one of the samples, and, if possible, several smears of different cell densities were made using the standard procedure. These smears were labeled "before syringing." The other samples were entrifuged, the original solutions were discarded, and 9 ml of the

solutions to be tested was added These samples were kept overnight at 4 C The following day the samples were syringed and smears were made as described above In experiments testing the effect of hyaluronidase ethanol was omitted from all solutions and the syringing was performed at 35 C

Cell density and percentage of single cells were calculated by counting cells in ten randomly chosen areas of 1 sq mm in the central part of the smear For the disaggregation experiments care was taken to ensure that only smears with the same cell density were compared

Results

Cell Disaggregation

There is a great variance in quality and cell composition among different cervical samples This depends on such variables as the age of the woman the day of the menstrual cycle inflammation and the use of contraceptives Therefore 22 widely varying cervical samples were processed for syringing tests (Table I) Comparing the nonsyringed samples with syringing in PBS 10% ethanol revealed that syringing leads to a considerable increase in the percentage of single epithelial cells up to a factor of 8 4 (case 3) The mean percentage of single cells achieved was 50%

In order to improve upon the syringing method the influences of EDTA and dithiothreitol present in the collection fluid were studied The experimental conditions were chosen in such a way that the procedure could be used later for large-scale applications Addition of 1mM or 5mM EDTA 6 5mM dithiothreitol and combinations of these did not lead to any further reproducible increase in the percentage of single cells

In Table II syringing in two different media was compared to syringing in PBS-10% ethanol These two media one solution according to the prescription of Escobar and co-workers[2] and one according to Husain and co-workers [4] have been reported to have a positive influence on cell disaggregation Syringing in the Escobar medium gave a higher percentage of single cells than in PBS-10% ethanol in five of the ten samples Although this looks quite promising three of these five cases showed severe cell damage Syringing in Husain s medium did not lead to any re-

Table I *Influence of Disaggregation Agents on Percentage of Single Epithelial Cells*

Sample no	Before syringing	Syringing in PBS-10% ethanol with: No additives	1mM EDTA	5mM EDTA	6 5mM dithiothreitol	1mM EDTA plus 6 5mM dithiothreitol	5mM EDTA plus 6 5mM dithiothreitol	Cell density (epithelial cells per sq mm)
1	27	53	53					56
2	10	47	49		45			50
3	5	42			36	30 (−)		32
4	20	47	36 (−)		31 ( )	50		65
5	*	58	56		52	50		48
6	10	19	30 (+)		38 (+)	33 (+)		58
7	31	51	57		53			66
8	26	75			70			53
9	25	71			66			68
10	13	49			41			22
11	*	54			61			52
12	41	72				39 (−)		22
13	48	58		43 (−)				49
14	35	62		62				55
15	4	14		22				38
16	21	20		47 (+)				38
17	26	59		55				53
18	28	57		34 (−)				59
19	26	36		40				59
20	28	46		45			49	59
21	20	45		42			54	64
22	19	51		45			39 (−)	76

*Cell density in the smear was too low to be compared to the other smears Decrease (−) or increase (+) in single cells is noted as compared to syringing in PBS 10% ethanol with no additives

producible improvement as compared to PBS-10% ethanol

The last disaggregating agent tested was the enzyme hyaluronidase in the presence or absence of 5mM EDTA (Table III) In the experiments done so far, no reproducible improvement to syringing in PBS without additives has been found

Table II *Influence of Disaggregation Media on Percentage of Single Epithelial Cells*

Sample no	Before syringing	Syringing in PBS-10% ethanol	Syringing in Escobar medium	Syringing in Husain s medium	Cell density (epithelial cells per sq mm)
1	28	46	47		59
2	19	51	55		76
3	26	59	49 (−)		56
4	12	40	14 ( )		49
5	12	66	55 (-)		59
6	21	20	50 (+)		38
7	28	57	69 (+)		59
8	26	36	58 (+)		59
9	20	45	63 (+)		64
10	12	40	62 (+)		48
11	27	53		53	56
12	22	75		61 (−)	53
13	26	62		31 (−)	60
14	23	54		66 (+)	52
15	10	19		3 (−)	58
16	*	70		66	54
17	*	82		73	24
18	*	51		64 (+)	24
19	*	79		76	30
20	*	32		41	42

*Not determined Escobar medium consists of 15mM KCl 50mM NaCl 16mM EDTA 40mM dithiothreitol and 135mM glycine (pH 6 8) †Husain s medium consists of 50mM KH_2PO_4 50mM NaCl 29 6mM NaOH 6 5mM dithiothreitol and 40% ethanol ‡ Decrease (−) or increase (+) in single cells is noted as compared to syringing in PBS 10% ethanol

Cell Deposition

Two methods of depositing the disaggregated cells onto a glass slide were compared (Table IV) In the first method, cells in solution were laid on a slide coated with polylysine (polylysine method) The second procedure is the carbowax method described above The experiment was done with five different samples, taking care that in each case the same number of cells was brought onto both slides In all cases a much better recovery of epithelial cells was found with the carbowax method In only one case was the recovery of leukocytes higher with the polylysine method The carbowax method is also much easier for routine use because smears can be air dried and stored for at least seven months without any morphologic damage

The deposition of the cells may lead to cell overlap by reaggregation or overcrowding For four cervical samples smears with different cell densities were made Plotting cell density against the percentage of single cells gave the results shown in Figure 5 In only one case was the ideal of 100% single epithelial cells found at a cell density of less than 6 cells/sq mm In the other cases, maximum percentages of single cells of 75% (cell density 11 cells/sq mm) 66% (cell density, 10 cells/sq mm) and 63% (cell density 19 cells/sq mm) were found In all cases there was a more-or-less-linear decrease in the percentage of single cells with increasing cell density

For the BioPEPR system the aim has been to prepare slides with a cell density of 50 cells/sq mm The cell distribution in the middle part of the smear has been evaluated automatically by the BioPEPR system Counting the number of epithelial cells in 6 000 fields of 6×8 mm taken from 1,000 slides gave the cell distribution shown in Figure 6 An average of 50

Table III *Influence of Disaggregation Agents on Percentage of Single Epithelial Cells*

Sample no	Before syringing	Syringing in PBS (35 C) with No additives	5mM EDTA	Hyaluronidase (1 mg/ml)	5mM EDTA plus hyaluronidase (1 mg/ml)	Cell density (epithelial cells per sq mm)
1	54	73		74	65	59
2	21	70		67	54 (−)	54
3	33	56	42 (−)	51	49	55
4	23	41	37	46	42	51
5	39	49	46	47	46	52
6	4	36	30	40	27	50
7	12	21	27	32 (+)	19	54
8	13	59	23 (−)	34 (−)	34 (−)	51

Decrease ( ) or increase (+) in single cells is noted as compared to syringing in PBS with no additives

cells/sq mm was found, although there was considerable variation

Table IV *Cell Recovery with Two Deposition Methods*

Sample	Type of cell	Cells per sq mm: Polylysine method	Cells per sq mm: Carbowax method
1	Epithelial cells	1	20
	Leukocytes	160	81
2	Epithelial cells	2	57
	Leukocytes	58	85
3	Epithelial cells	5	68
	Leukocytes	10	15
4	Epithelial cells	7	25
	Leukocytes	27	41
5	Epithelial cells	10	37
	Leukocytes	0	0
	Bare nuclei	0	7

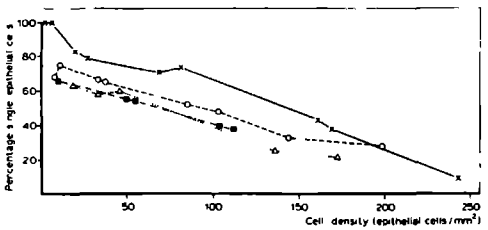

Figure 5
Percentage of single cells plotted against cell densities for four different cervical samples (× ○ △ ■)

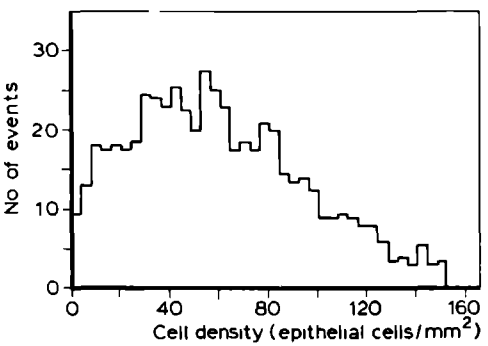

Figure 6
Frequency distribution of cell density per square millimeter from 6 000 fields measured on BioPEPR

Discussion

The preparation procedure described above has been developed with the intention of preparing thousands of specimens in one year in order to monitor the effectiveness of the BioPEPR system in a large-scale field test [19] In practice, an average of 80 and a maximum of 120 specimens per day have been processed, making it possible to analyze more than 10 000 smears per year

Much of the handling has been simplified and automated The same container is used from sample collection to deposition of the cells on the slide The syringing the addition of the correct carbowax volume and the cell deposition have been automated other handling is kept to a minimum Complex preparation procedures[5] or laborious preparation steps such as specimen enrichment on a density column,[8 13] have not been tried because these techniques will be impractical in a large-scale semiautomatic or fully automatic procedure

All steps in the preparation procedure have been critically studied The syringing procedure is effective and yields an average of 50% single cells (see Tables I to III) The method introduces very few artifacts Cells from cervical samples that are quite fragile owing to cytolysis and inflammatory conditions did not show much destruction after syringing A greater clustering of leukocytes was found in some cases these clusters can be easily recognized by the BioPEPR system [18] The only difficulty the syringing process introduces is that the diagnosis carcinoma *in situ* is more difficult to make as a result of the dispersal of the diagnostically important cells This is a general problem of all disaggregation techniques

The composition of the collection fluid has been kept very simple No improvement to the syringing was seen when various reagents were added (Tables I to III) In addition, the high level of preservation of cell morphology using the more simple solution makes it unnecessary to develop a more complex medium

The carbowax method has been used for cell deposition, offering better cell recovery and easier handling than the polylysine technique This method also has an advantage when more than one smear is made from a scrape These extra smears can be stored and later fixed and stained for use in other experiments The method introduces no morphologic changes Occasional damage to fragile leukocytes has been noted, but they still can be easily recognized

A compromise between the requirements of sufficient cell density and a certain minimum percentage

of single cells for accurate diagnosis has to be found for every image analysis system Tanaka and co-workers[11] found a cell density of less than 50 cells/sq mm to be best suited to the CYBEST system A cell density of 50 cells/sq mm has been arbitrarily chosen for the BioPEPR system,[18] giving a false-alarm rate of 24 6% and a missed-positive rate of 3 5% [19] Although the preparation procedure results in a mean cell density of 50 cells/sq mm, a degree of variation is found (Figure 6) There are several reasons for this Using the Hemaprep for spreading the cells, cell density gradually increases in the direction of motion Variations may also occur in the number of cells brought onto the slide due to fluctuations in the volume of carbowax added or to counting errors made by the Coulter Counter These counting errors are inherent in a cervical sample and are caused by the variation in cell integrity, the presence of artifacts (cell debris, protein, noncellular material) and the difficulty in finding a size threshold between the smaller epithelial cells (columnar and parabasal cells) and leukocytes However, a large number of small epithelial cells in the sample in general will not lead to more cell overlap An alternative counting method is that described by Bahr and co-workers[1] using light scatter and absorption The authors report that such a system is less sensitive to counting errors caused by leukocytes, but the other problems mentioned here remain

Figure 5 shows that a relatively large change in cell density from the nominal 50 cells/sq mm does not markedly affect the percentage of single cells The diagnostic accuracy of BioPEPR will therefore not be significantly altered Experiments are underway to monitor the effect on false-alarm and missed-positive rates of increasing the cell density from the present 50 cells/sq mm

Of 3,400 smears prepared from second scrapes according to this standard procedure, 5 2% have been rejected by the BioPEPR system as containing too few epithelial cells [19] Tanaka and co-workers,[10] using a similar criterion, found 1 4% of the smears prepared for the CYBEST system to be inadequate

Current research is now focused on fully automating the entire preparation procedure, from syringing to smear preparation A simpler and faster method for syringing is now being investigated, as is a deposition method that lays down the cells directly onto the slide from the original cell suspension This will eliminate the centrifugation step and change of solution, which are difficult to automate. All these methods have to be implemented in one fully automatic system that is capable of handling at least 200 cervical samples a day

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SECTION 2.2

A new disaggregation device for cytology specimens

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A New Disaggregation Device for Cytology Specimens[1]

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A new means of disaggregating cytology specimens in suspension using an immersible rotor device is described. The new rotor is compared to an automated syringing apparatus using cervical samples. Similar results using both devices are obtained for both normal and abnormal specimens.

Key terms: Cytology, specimen preparation, automation

A prerequisite for the (pre)screening of cytology specimens by an automated image analysis system is a reproducible and practical method for preparing smears of disaggregated cell suspensions Many procedures have been described (1,4,10,14,16,17), but most are limited in that they are labor-intensive or very time consuming To make automated cytology an attractive addition to routine manual cytology, the specimen should be handled as little as possible, and the processing time should certainly not be much longer than the time needed to prepare a cervical smear with conventional manual methods

The Biological Precision Encoding and Pattern Recognition (BioPEPR) project (19) is concerned with full automation of cervical smear prescreening, including the preparation phase In a previous paper (10), we described a semiautomatic procedure by which about 100 cervical smears a day could be prepared Because it proved difficult to fully automate this entire process as it was, certain steps in the process were reevaluated in order to make a fully automated system feasible

All of the automated disaggregation systems developed to date have been automated versions of the syringing procedure (1,4,7,9,10,16) With syringing it is difficult to fully automate the changing of the needles or the syringes, and to provide adequate rinsing of the device between samples to prevent specimen contamination

The present paper describes a new method of disaggregating cell suspensions Cell disaggregation with this device, a specially designed rotor, is easy to automate and has proved to be efficient In this paper the rotor is described, and results obtained with this rotor when disaggregating cervical specimens are presented

MATERIALS AND METHODS

Cervical cells were collected as second or third scrapes using a plastic spatula that was rinsed in a preservative phosphate buffered saline solution, containing 20% ethanol Disaggregation was performed either by a syringing technique using a peristaltic pump device (10) for 15 min with 19 gauge needles and a speed of 55 ml/min, or by a rotor immersed in the sample vial containing 9 ml of cell suspension (Fig 1A) For comparative studies the original sample was divided before disaggregation into two to four sample containers, and the fluid volume was adjusted back to 9 ml

After disaggregation the cells were deposited onto glass microscope slides, as described elsewhere (10)

The rotor device used consists of a 24 V DC Maxon motor (Sachseln, Switzerland) with a vertical axle on which different cylindrical rotor heads can be mounted The distance from the outer edge of the rotor head to the vessel wall is about 2 mm Figure 1B shows two examples of hollow rotor heads, both having an external diameter of 17 mm and an internal diameter of 10 mm In one of the rotors (right in the figure), 1 mm diameter holes were drilled at an angle of 30° with respect to the tangent of the outside surface (in the direction of rotation) The holes were thought to provide an action similar to that of syringing Experiments were performed to compare the two rotor heads During use, the rotor was driven at a speed of 6,000 rpm Usually, samples were disaggregated for 30 s Shorter (15 s) as well as longer (45 s) times were also investigated After disaggregation, the rotor was spun for a few seconds above the cell suspension to eliminate possible remaining fluid and cells Thereafter it was rinsed in tap water by spinning

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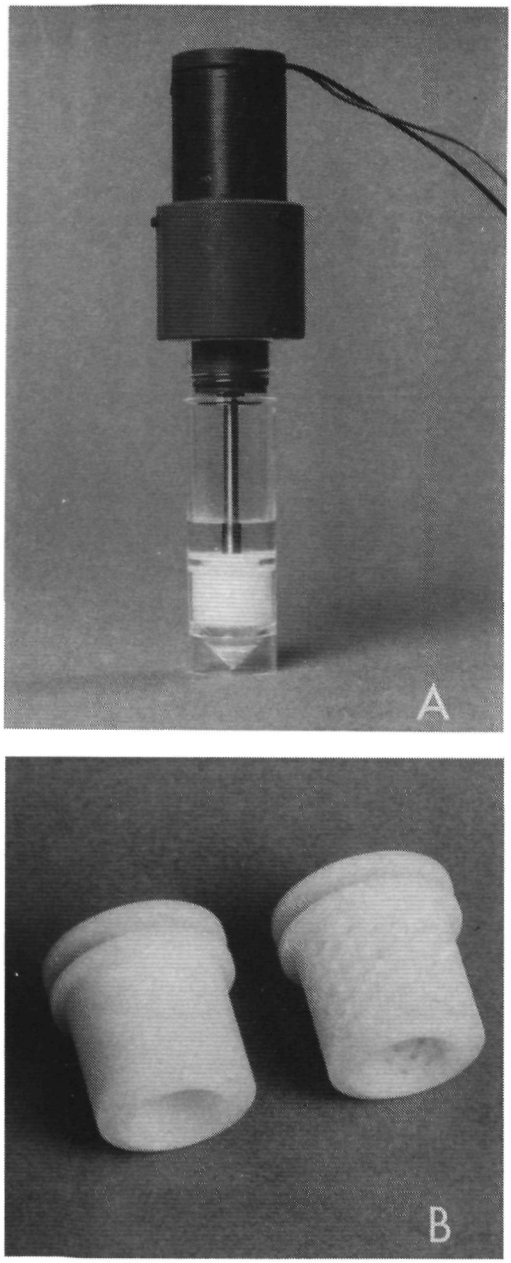

FIG. 1. A) Immersible rotor system for the disaggregation of cytology specimens. B) Two different rotors, which can be mounted on the device in A. The left rotor without holes; the right rotor contains 1-mm holes. For further details see the text.

for about 5 s and spun above the fluid level to remove excess water.

The effectiveness of cell disaggregation achieved by the different techniques was evaluated by counting the number of singly lying epithelial cells, the total number of epithelial cells, including all cells in aggregates such as sheets and clusters, and the total number of leukocytes in the central part of the smear. Singly lying epithelial cells were defined as cells that did not touch or overlap other epithelial cells. Since leukocytes are rather fragile cells, they were counted to see if the disaggregation resulted in a severe destruction of this cell type. Together with the counting procedure the quality of the disaggregated cells was assessed. Although the cell deposition procedure resulted in a certain number of overlapping cells, no attempts were made to discriminate these overlaps from original aggregates, because criteria to distinguish these were difficult to formulate. Counting was done manually under a microscope in ten areas of $1mm^2$ (16× objective field in the microscope) each that were sampled in a regular pattern. The central area was chosen because there is an increase in cell overlap at the beginning and end of the slide owing to the cell deposition procedure (analogous to the "wedge technique," see reference 10).

The reproducibility of the counting procedure was tested in two ways, by repeating the count on different fields of the same slide and by making counts on two different slides made from the same cervical sample.

Using a number of normal samples, a study was made to compare the effectiveness of the syringing technique to that of the rotor device, and to compare the results of the rotor for times of 15 s, 30 s, and 45 s.

For a number of abnormal samples, the number of singly lying cells and total numbers of abnormal cells were counted in a 2 × 2-cm area in the central part of the slide. Abnormal cells were defined as cells consistent with a slight dysplasia or a more severe epithelial abnormality. Singly lying abnormal cells were defined as abnormal cells that did not touch or overlap other normal or abnormal epithelial cells. A comparison of the syringing technique to the rotor device was made, again using three different rotor running periods. In another test a series of 40 "abnormal slides" was evaluated to determine the percentage of singly lying abnormal cells as a function of the degree of abnormality. In this last test all samples were disaggregated using the rotor device for 30 s.

The results of statistical analysis of the data are shown in the Tables 1–4. A regression analysis (15) was performed to compare the results obtained by repeated counting (Table 1), to compare the results of the syringing technique to the results of the rotor (Tables 2,3), and to compare the results of one rotor type to the results of the second rotor type (Table 4).

RESULTS

Counts from the reproducibility study are presented in Table 1. Both the repeat counts from same sample

and the counts from duplicate slides show good agreement

Table 2 shows the comparison between two different disaggregation procedures for the normal samples sy ringing for a period of 15 min, and treatment with the rotor with 1 mm holes for three different periods of time Extensive studies elsewhere (1,4,10,16) had proven that the syringing method yielded a considerable increase of single cells, compared to nonsyringed samples, without additional significant cell loss or morphological cell damage In previous studies in our laboratory syringing was shown to increase the percentage of single cells counted on the slide from an average of 23% to an average of 50% (10) Because the effectiveness of syring ing had already been shown, and because the samples used in this comparative study were typical of those used in our previous study, "nonsyringed" data are not presented here

As can be seen in Table 2, no significant difference is noted between the number of single cells, the total num ber of cells, and the number of leukocytes, whether treated with the rotor or the syringing device Also no significant difference is noted between the different du rations of rotor treatment A minimal time of 15 s re sulted in optimally disaggregated cell samples

The same experiment was also performed on a variety

Table 1
Reproducibility of the Cell Counting[a]

	Same slide, different fields				Same sample, different slides			
	Epithelial cells/mm^2		% single epithelial cells		Epithelial cells/mm^2		% single epithelial cells	
Sample No	First count	Second count	First count	Second count	Slide A	Slide B	Slide A	Slide B
1	116	114	39	33	63	66	41	40
2	108	114	36	37	95	98	32	37
3	117	108	41	34	61	62	53	46
4	50	79	42	28	78	79	31	37
5	95	81	58	60	69	67	35	38
6	52	60	64	64	117	113	38	37
7	67	72	67	64	114	121	25	21
	r = 0 893 P<0 01		r = 0 948 P<0 01		r = 0 989 P<0 001		r = 0 845 P<0 05	

[a] r, coefficient of correlation, P, level of significance

Table 2
Comparison of an Automated Syringing Apparatus With the Rotor on Normal Cervical Samples[a]

	% single epithelial cells				Epithelial cells/mm^2				Leukocytes/mm^2			
		Rotor treatment				Rotor treatment				Rotor treatment		
Sample No	Syringing	15 s	30 s	45 s	Syringing	15 s	30 s	45 s	Syringing	15 s	30 s	45 s
1	67	61	89	77	48	59	38	44	8	13	15	11
2	44	52	54	46	33	33	42	48	10	12	12	10
3	74	78	66	64	21	18	40	56	9	5	13	10
4	59	40	34	51	22	26	22	27	1	0	0	0
5	81	66	68	71	36	49	45	44	3	4	3	3
6	66	42	38	48	30	43	50	46	12	13	11	11
7	45	44	38	53	54	48	65	48	34	18	32	22
8	58	81	78	76	49	44	37	29	16	19	39	15
9	81	92	80	79	47	44	47	44	3	2	4	3
10	62	74	62	68	47	56	47	40	11	23	16	16
11	76	71	73	75	58	50	39	40	12	14	12	15
12	65	73	76	72	44	41	37	36	15	23	15	15
13	73	64	58	68	42	49	51	37	5	11	14	8
14	83	76	73	76	26	27	42	35	28	39	46	36
15	54	64	67	60	96	77	63	86	17	37	46	42
16	57	45	62	54	32	24	26	27	5	12	9	10

[a] % single epithelial cells, syringing versus 15 s rotor treatment r = 0 588, P < 0 05, % single epithelial cells, 15 s versus 30 s rotor treatment r = 0 514, P < 0 05, % single epithelial cells, 15 s versus 45 s rotor treatment r = 0 842, P < 0 001, epithelial cells/mm^2, syringing versus 15 s rotor treatment r = 0 872, P < 0 001, epithelial cells/mm^2, 15 s versus 30 s rotor treatment r = 0 629, P < 0 01, epithelial cells/mm^2, 15 s rotor treatment r = 0 703, P < 0 01, leukocytes/mm^2, 15 s versus 30 s rotor treatment r = 0 868, P < 0 001, leukocytes/mm^2 15 s versus 45 s rotor treatment r = 0 941, P < 0 001 r, coefficient of correlation, P, level of significance

Table 3
Comparison of an Automated Syringing Apparatus With the Rotor on Abnormal Cervical Samples[a]

	% single abnormal cells				Total No of abnormal cells				Epithelial cells/mm^2			
		Rotor treatment				Rotor treatment				Rotor treatment		
Cytology	Syringing	15 s	30 s	45 s	Syringing	15 s	30 s	45 s	Syringing	15 s	30 s	45 s
Slight dysplasia	35 0	28 5	–	–	20	35	–	–	73 2	62 2	–	–
Slight dysplasia	3 8	9 7	5 4	15 0	159	93	56	60	103 3	76 5	91 3	98 8
Slight dysplasia	22 2	18 1	14 3	8 2	27	33	28	49	64 0	47 5	54 3	63 2
Slight dysplasia	33 3	27 0	12 9	–	48	37	62	–	40 3	17 5	35 5	–
Slight dysplasia	5 0	4 9	5 7	4 5	60	61	88	67	62 8	84 8	88 3	79 2
Slight to moderate dysplasia	34 6	39 1	30 0	37 5	26	23	40	24	45 3	43 3	42 8	51 8
Moderate dysplasia	14 2	19 4	21 4	10 2	14	31	28	49	15 2	21 8	11 0	23 7
Moderate dysplasia	50 6	31 4	–	–	77	102	–	–	45 8	69 2	–	–
Moderate dysplasia	34 0	25 0	–	37 0	55	146	–	119	40 0	65 1	–	55 8
Moderate dysplasia	9 0	24 0	35 0	21 0	207	251	311	324	23 1	27 2	37 9	43 8
Severe dysplasia	17 3	17 1	17 6	15 5	369	404	427	245	44 0	64 2	56 3	44 7
Severe dysplasia	4 8	6 0	7 0	8 1	272	386	345	321	56 2	63 8	66 0	72 3
Severe dysplasia	37 0	32 0	–	33 0	696	815	–	675	23 2	20 2	–	20 8
Severe dysplasia/CIS	28 0	25 0	–	–	64	71	–	–	70 8	71 9	–	–
CIS	25 1	23 0	22 6	–	351	357	381	–	72 3	62 5	50 2	–

[a]% single abnormal cells, syringing versus 15 s rotor treatment $r = 0\,850$, $P < 0\,001$, % single abnormal cells 15 s versus 30 s rotor treatment $r = 0\,731$, $P < 0\,05$, % single abnormal cells, 15 s versus 45 s rotor treatment $r = 0\,859$ $P < 0\,01$, total No of abnormal cells, syringing versus 15 s rotor treatment $r = 0\,982$, $P < 0\,001$, total No of abnormal cells 15 s versus 30 s rotor treatment $r = 0\,982$, $P < 0\,001$, total No of abnormal cells, 15 s versus 45 s rotor treatment $r = 0\,971$, $P < 0\,001$ total No of epithelial cells, syringing versus 15 s rotor treatment $r = 0\,730$, $P < 0\,01$, total No of epithelial cells, 15 s versus 30 s rotor treatment $r = 0\,903$, $P < 0\,001$, total No of epithelial cells, 15 s versus 45 s rotor treatment $r = 0\,851$, $P < 0\,01$ r, coefficient of correlation
P, level of significance
CIS, carcinoma in situ

Table 4
Comparison of Two Different Types of Rotors Used on Normal Cervical Samples[a]

	% single epithelial cells		Epithelial cells/mm^2		Leukocytes/mm^2	
Sample No	Rotor with 1 mm holes	Solid rotor	Rotor with 1 mm holes	Solid rotor	Rotor with 1 mm holes	Solid rotor
1	50	55	120	187	6	4
2	28	28	62	124	0	0
3	68	78	89	52	22	23
4	52	40	69	79	20	13
5	42	48	70	63	2	7
6	47	45	20	23	0	0
7	52	59	100	86	20	14
8	40	40	129	140	175	138
9	57	56	64	63	12	11
10	52	48	46	49	1	1
	$r = 0\,886$ $P < 0\,001$		$r = 0\,769$ $P < 0\,01$		$r = 0\,998$ $P < 0\,001$	

[a]r, coefficient of correlation
P, level of significance

of abnormal cervical specimens (Table 3) Special attention was given to both disaggregation procedures with regard to their influence on abnormal cells Again no significant differences concerning the number of singly lying abnormal cells as well as the total number of abnormal cells were observed between the syringing and the rotor procedure Also for abnormal cells a minimal time of 15 s resulted in optimal disaggregation

In Table 4, the results of a comparison of two different types of rotors are given, one rotor with 1 mm holes, and one without holes No significant differences were observed between the results obtained with these two different rotors

The rotor with 1 mm holes was used in a large scale screening study, for the routine preparation of 5,500 cervical slides made from second scrapes After cytologic evaluation to determine abnormality, 40 slides were chosen from this material containing cells consistent with varying degrees of epithelial abnormalities, and the number of singly lying abnormal cells was determined As Table 5 shows, the proportion of singly lying abnormal cells remained fairly constant, but the total

Table 5
Performance of the Rotor in Disaggregation of Abnormal Cells[a]

Cytology	No of samples	% single abnormal cells	Total No of abnormal cells per slide	Epithelial cells/mm^2
Slight dysplasia	10	31 ± 14	298 ± 164	52 ± 22
Moderate dysplasia	10	22 ± 14	400 ± 292	48 ± 15
Severe dysplasia	10	33 ± 23	526 ± 348	48 ± 22
Carcinoma in situ	10	25 ± 16	790 ± 542	45 ± 35

[a]Numbers shown are mean ± standard deviation

number of abnormal cells counted in each sample increased with the increasing degree of abnormality of the cell sample

DISCUSSION

The syringing technique works by exerting a shear force at the tip of the needle that breaks clusters of cells preferentially along the natural boundaries between cells The syringing method is difficult to fully automate, however, primarily because of inadequate rinsing, which causes specimen contamination, and because of the necessity to frequently change the needles or the syringes The need for an equally effective, yet more automatically workable disaggregation technique led to the development of the rotor device described here

The rotor system also makes use of shear forces Here, a circumferential shear force is formed in the cell suspension layers adjacent to the rotor head, owing to the rotation of the cylinder, the viscosity of the fluid, and the size of the cellular clumps Larger clusters are exposed to a gradient of fluid velocity extending from the rotor to the inner vessel wall, providing the force to tear the clusters along the path of least resistance at their natural boundaries The irregular shape and size of the clusters ensures the oscillating movement of the clusters into the higher level forces close to the rotor head

The rotor head can be constructed as a simple cylinder that is easy to clean, eliminating cell contamination from one specimen to another As shown in Table 4, the use of holes drilled through the cylinder (which were thought to induce a syringing effect) did not improve disaggregation The circumferential shear force seems to be the dominant effect The rotor also works considerably more rapid than a typical syringing system—15 s proved to be adequate For the automated syringing system used in this study 15 min were required (10), whereas for other automated versions 2 (16) to 3 (1) min have been reported

From the presented data it may be concluded that the rotor device gives very similar disaggregation results as the syringing device This finding was consistent for a wide variety of cervical specimens, both normal and abnormal Using both methods, no differences in cell morphology of the squamous epithelial cells were seen, whereas the same number of the more fragile leukocytes was found (see Table 2) Other studies (11) in which the rotor device has been used show that the morphology of columnar epithelial cells and other more fragile epithelial cells is well preserved It should be noted that both disaggregation techniques were designed for use in preparing slides for an image analysis system Such a system is tolerant of a fair number of touching or slightly overlapping cells Observations indicate that the size and numbers of the remaining clusters might be an additional parameter in the further classification of atypical or abnormal specimens It is possible that a greater degree of disaggregation could be obtained through higher speed rotation or modification of the rotor head, or perhaps through the use of (bio)chemical methods (8) The rotor has not been tested at higher speeds Increasing the shear force might result in cell damage, whereas the gain in rotating time is not relevant A very high rotational speed of the rotor head should provide results similar to those of the more forceful syringing technique now used in such cases (7,9) No further attempts to disaggregate cell clumps by (bio)chemical means have been made, since these procedures generally fail or result in severe aspecific cell damage (5,6,8,10,18)

It is important, of course, to determine how any disaggregation device will work on abnormal cells In our study using abnormal samples, the rotor device again performed very similarly to the syringing apparatus For abnormal cells, a lower percentage of singly lying cells was found than for normal cells In general only 22–33% of the abnormal cells were disaggregated, compared to 60% of the normal ones Again, an automated image analysis system should be capable of recognizing many of these abnormal cells, even when clustered, since good nuclear information is generally still available, and simple algorithms are capable of identifying cells with overlapping cytoplasms (20) It is of interest to note that the fraction of abnormal cells increased with samples of increasing abnormality (see Table 5), this same tendency has been reported elsewhere for conventional smears made from first scrapes (2,3 12,13) For slight dysplasia abnormal cells constituted 0 75% of the total number of epithelial cells, whereas for carcinoma in situ a fraction of 2 2% was found

In the study described here a new rotor disaggregation device was used in the preparation of cervical cytology specimens The use of this method with other cytological

material such as sputum, ascitic and pleural fluids is clearly possible Preliminary results with sputum indi cate results similar to those achieved with cervical scrapes (data not reported here) The rotor disaggrega tion technique seems, therefore, well suited to a variety of clinical and research applications in which disaggre gated cell suspensions are required

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SECTION 2.3

Pressure-fixation method of transferring cells from polycarbonate filters to glass slides

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Pressure-Fixation Method of Transferring Cells from Polycarbonate Filters to Glass Slides

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A technique for the preparation of cytology slides is presented by which cells collected on a polycarbonate membrane filter are transferred to a slide by means of simultaneous pressure and fixation. Using cervical samples as a model, the influence of the filtration rate, filter pore size and duration of pressure application on cell recovery was analyzed. The present version of the preparation procedure uses manual techniques that define the operating characteristics of a fully automated procedure.

Cytologic preparations from such body sites as the uterine cervix, urine, sputum and body fluids are characterized by a nonuniform distribution of cells, with frequent dense clumping of diagnostically important cells. This inhomogeneity and clumping render individual cell examination difficult for conventional light microscopic interpretation and make automated smear screening virtually impossible.

Numerous techniques have been developed for obtaining monodispersed suspensions of cells from cytology specimens.[1,3,8-10,14,16] The method that optimally disaggregates cells with a minimum of cell loss or damage seems to be syringing or some variation of it.[8,9,14] These monodispersed specimens are suitable without further processing for flow cytometric analysis.[4,5] However, deposition and retention of cells on slides for light microscopic examination or as input to image-processing instruments remain a problem. Several solutions to this problem have been proposed, including centrifugation implemented in various ways,[1,2,7,14,17] sedimentation of cells directly from suspension onto slides[6,10] and collection of cells on a filter followed by transfer of the cells to a slide.[13,15] Most of these methods have been both

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labor intensive and difficult to implement in a high-volume, automated system, thus nullifying any benefit of intended automated prescreening.

This report describes a manual method, based on filtration, of depositing monodispersed cell suspensions on slides, resulting in preparations with evenly distributed cells of good morphology. Certain important parameters in the collection and transfer process were critically evaluated, and the implementation of this method into a fully automated system is discussed.

Materials and Methods

Cervical cells were collected with a plastic spatula, suspended in phosphate-buffered saline (PBS) containing 20% ethanol as a preservative[10] and stored at 4 C. One to three days after collection the cells were disaggregated by hydrodynamic shear forces induced by a rotor device, as described elsewhere.[12] Cell concentration was determined using a Coulter model ZBI (Coulter Electronics, Hialeah, Florida), and cell dilutions to a final volume of 9 ml were made with PBS-20% ethanol, with a cell concentration of approximately 4–8 $\times$ 10^3 epithelial cells per milliliter. For comparative studies up to four cell dilutions were made from one cervical sample.

A filtration assembly (Millipore, Bedford, Massachusetts) was used to collect the cells on 19$\times$42-mm polycarbonate filters (Nuclepore, Pleasanton, California). The characteristics of these filters (flat and smooth with capillary pores) were such that the cells could easily be transferred to slides during the later application of pressure. The filtration assembly consisted of an Erlenmeyer flask connected to a vacuum pump, a fritted glass filter support on top of it and a stainless steel funnel with a rectangular opening (18$\times$36 mm) that covered the filter. The filters were placed on the support, which had been premoistened with a few milliliters of PBS-20% ethanol to evenly flatten the filter. The funnel was then placed over it. Support, filter and funnel were held together by a spring clamp. The cell suspension (approximately 36–72 $\times$ 10^4 cells) was pipetted onto the filter and aspirated through with minimal vacuum (-1 cm water pressure), unless otherwise stated. This procedure resulted in a filtration rate of approximately 12 ml/min. Immediately after the fluid had run through, the vacuum was disconnected and the filter taken from the support and placed cell side down onto a standard glass microscope slide. To the top of the filter was applied a polyether sponge (52$\times$27$\times$10 mm; Draka Interfoam, Hillegom, the Netherlands),

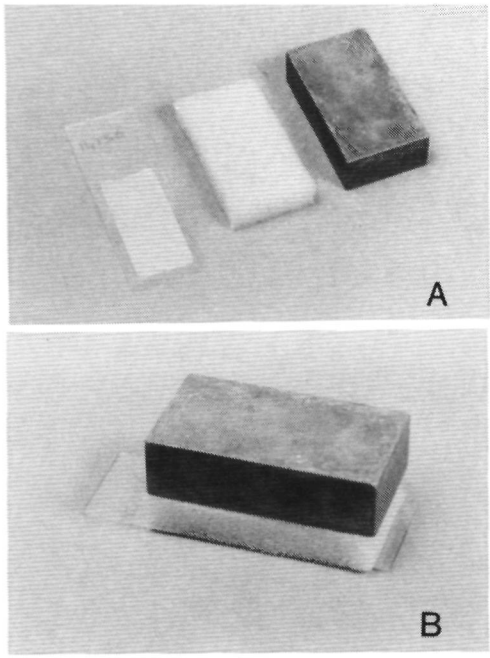

Figure 1
(A) Components of the cell transfer procedure. From left to right: slide with polycarbonate filter, polyether sponge and brass weight. (B) Cell transfer from filter to glass slide. From top to bottom: brass weight, polyether sponge moistened with fixative and slide. The filter with the cells (not visible) is present between the sponge and the slide.

previously moistened with 1 to 2 ml of ethanol or a methanol-37% formaldehyde solution-acetic acid mixture (85:10:5, v/v/v; MFA). This fixative was used when thionine-Feulgen-Congo red staining[11] was to be carried out. A 210-gm brass weight (55$\times$30$\times$15 mm) was then gently placed on top of the sponge for 15 to 60 seconds (Figure 1). This procedure transferred and fixed the cells to the glass slide. The weight and sponge were then removed and the filter peeled off. The slide, as well as the filter (after attachment to another glass slide), were immersed in the same fixative for an additional 30 minutes. The preparations were then stained with either the Papanicolaou or thionine-Feulgen-Congo red technique.[11]

The number of epithelial cells found on the slide and the filter was calculated from counting 24 1-sq-mm fields sampled in a regular pattern and expressed

Table I *Number of Cells Found on Slide and Filter with Fixation Either Following or During Application of Pressure*

		Number of epithelial cells/sq mm				Percentage of total epithelial cells counted on slide and filter			
		Mean	S.D.	S.E.	Range	Mean	S.D.	S.E.	Range
Pressure followed by fixation	Slide	45 8	9 2	3 8	31 2-58 4	99 2	1 2	0 5	96 9-100 0
	Filter	0 4	0 5	0 2	0 0-1 3	0 8	1 2	0 5	0 0-3 1
Simultaneous pressure and fixation	Slide	66 4	10 8	4 4	58 2-78 8	99 2	0 8	0 3	97 5-99 9
	Filter	0 5	0 5	0 2	0 1-1 4	0 8	0 9	0 4	0 1-2 5

Number of cervical samples tested 6
Filter pore size 8μm
Filtration rate 12 ml/min
Pressure time 30 seconds
Fixative ethanol

S D = standard deviation
S E = standard error

as cells per sq mm. In most cases the total number of cells left on the filter was so low (less than 500) that the entire filter was counted.

The number of cells that passed through the filter during filtration was determined by collecting the filtrate in a plastic centrifuge tube. The filtrate was centrifuged for ten minutes at 2,300 g, the supernatant was then discarded, and 35μl of 2% polyethylene glycol 1500 (Carbowax) in 50% ethanol was added to the resulting pellet. The pellet was resuspended by vortexing, with the resulting sample transferred to a glass slide by means of a plastic pipet, air dried, ethanol fixed and Papanicolaou stained. All cells present on these slides were counted and expressed as cells per square millimeter, corrected to the original filter area

Results

The original work from which the present study was derived[15] used a plastic sheet in place of the sponge between the weight and the filter and fixed the cells to the slide following application of pressure rather than simultaneously with the pressure step. Thus, the first study carried out in the present work was a comparison of fixation during pressure application with fixation following the application of pressure. Table I shows the number of cells found on the slides and filters for both techniques. Testing the difference between recoveries on the slides for each sample, the simultaneous pressure and fixation gave significantly higher cell recovery (Student's t-test, $p<0.005$). Cells were presumably lost during fixation or staining when using the technique of pressure followed by fixation. There was no significant difference between the methods in the cell loss by adherence to the filters. Thus, simultaneous pressure and fixation was adopted as the standard procedure for the remaining experiments.

Additional parameters that could influence the ultimate cell recovery were analyzed further: filtra-

Table II *Effect of Varied Filtration Rate on Number of Cells Found on Slide on Filter and in Filtrate*

		Number of epithelial cells/sq mm				Percentage of total epithelial cells counted on slide, on filter and in filtrate			
Filtration rate (ml/min)		Mean	S.D	S E	Range	Mean	S.D.	S E.	Range
9	Slide	84 6	13 7	6 1	66 2-101 0	98 3	0 7	0 3	97 1-98 9
	Filter	1 2	0 5	0 2	0 6 1 3	1 3	0 6	0 3	0 6-2 3
	Filtrate	0 3	0 3	0 1	0 0-0 6	0 4	0 3	0 1	0 0-0 6
13 5	Slide	71 6	4 1	1 8	67 5-77 8	98 4	0 8	0 4	97 6-99 6
	Filter	0 8	0 4	0 2	0 3-1 2	1 1	0 5	0 2	0 4-1 6
	Filtrate	0 4	0 3	0 1	0 0-0 7	0 5	0 4	0 2	0 0-0 1
27	Slide	80 3	16 3	7 3	67 5-108 6	97 5	1 4	0 6	96 2-99 6
	Filter	1 8	1 3	0 6	0 3-3 7	2 1	1 2	0 5	0 4-3 3
	Filtrate	0 3	0 3	0 1	0 0-0 8	0 4	0 4	0 2	0 0-1 1

Number of cervical samples tested 5
Filter pore size 5μm
Pressure time 15 seconds
Fixative ethanol

Table III *Number of Cells Found on Slide on Filter and in Filtrate After Using Polycarbonate Filters of Different Pore Diameters*

Pore diameter (μm)		Number of epithelial cells/sq mm				Percentage of total epithelial cells counted on slide, on filter and in filtrate			
		Mean	S.D.	S.E.	Range	Mean	S.D.	S E.	Range
5	Slide	120 0	19 0	7 8	92.0–150 0	99 7	0 3	0 1	99 2–99 9
	Filter	0 2	0 1	<0 1	0 1–0 4	0 2	0 1	<0 1	0 1–0 3
	Filtrate	0 3	0 3	0 1	0 0–0 9	0 2	0 2	0 1	0 0–0 7
8	Slide	113 0	35 0	14 3	80 0–154 0	99 6	0 2	0 1	99 4–99 8
	Filter	0 3	0 2	0 1	0 1–0 6	0 3	0 1	<0 1	0 1–0 4
	Filtrate	0 1	0 1	<0 1	0 0–0 3	0 1	0 1	<0 1	0 0–0 3

Number of cervical samples tested 6
Filtration rate 12 ml/min
Pressure time 60 seconds
Fixative MFA

tion rate, filter pore size and duration of pressure application. In addition, data on cell recovery, cell distribution and the reproducibility of the method have been obtained.

Filtration Rate

By changing the vacuum pressure, filtration rates of 9 to 27 ml/min were obtained. The minimum rate tested (9 ml/min) was close to that achieved without use of a vacuum. As Table II shows, no differences in cell recovery were observed between the three filtration rates. In each case more than 96% of the epithelial cells was recovered on the glass slide, with less than 4% of the cells found in the filtrate or on the Nuclepore filter. The differences found in absolute cell recovery (cells per square millimeter) might have been because of variations in the number of cells in the aliquots before filtration.

Filter Pore Size

Table III presents the results of varying filter pore size. Only filters of 3μm, 5μm and 8μm pore size were tested since the filtration rate achieved with those filters was acceptable. There was no difference in cell recovery rates among the different filters. However, filters with 3μm pores retained far more cell debris and protein precipitate than did those with 5μm and 8μm pores. Thus, the filters with 3μm pores were not studied further.

Pressure Time

To evaluate the duration of pressure application during fixation, four durations were tested. 15, 30, 45 and 60 seconds. With all four times tested, greater than 98% recovery on the slides was achieved (Table IV).

Recovery of Atypical and Dysplastic Cells

In order to gain some preliminary insight into the possible selective loss of atypical and/or dysplastic cells using the pressure-fixation technique, six specimens were evaluated as described in Table V. To achieve statistically representative numbers of abnormal cells, a 2×2-cm field (400 sq mm) was counted in place of the 24 1-sq-mm fields used in the other studies. Although the number of samples was far too

Table IV *Effect of Varied Pressure Time on the Number of Cells Found on Slide and Filter*

Pressure time (seconds)		Number of epithelial cells/sq mm				Percentage of total epithelial cells counted on slide and filter			
		Mean	S D	S E.	Range	Mean	S.D.	S E.	Range
15	Slide	87 5	8 6	3 8	77 3–98 9	99 6	0 2	0 1	99 4–100 0
	Filter	0 3	0 2	0 1	0 0–0 5	0 4	0 2	0 1	0 0–0 6
30	Slide	83 9	9 0	4 0	75 2–99 1	99 6	0 2	0 1	99 3–99 9
	Filter	0 4	0 2	0 1	0 1–0 7	0 4	0 2	0 1	0 1–0 7
45	Slide	86 6	8 1	3 6	79 0 97 2	99 3	0 7	0 3	98 2–100 0
	Filter	0 6	0 5	0.2	0 0–1 4	0 7	0 7	0 3	0 0–1 8
60	Slide	81 8	4 8	2 1	75 2–88 2	99 5	0 3	0 1	99 2–99 9
	Filter	0 4	0 2	0 1	0 1–0 6	0 5	0 3	0 1	0 1–0 8

Number of cervical samples tested 5
Filter pore size 5μm
Filtration rate 12 ml min
Fixative ethanol

Table V *Number of Normal and Atypical/Dysplastic Cells Found on Slide, on Filter and in Filtrate After Using the Pressure-Fixation Technique*

		Number of epithelial cells/sq mm					
		Normal			Atypical/dysplastic		
Sample no.	Cytology	Slide	Filter	Filtrate	Slide	Filter	Filtrate
1	Inflammatory atypia	129	0 1	<0 1	0 3	0 0	0.0
2	Inflammatory atypia	114	0 1	0 9	4 3	0 0	0.0
3	Atypia	119	0 1	0 2	31 2	0 1	0.0
4	Slight dysplasia	81	0 1	N D.	11 0	0 0	N D
5	Moderate to severe dysplasia	106	0 1	N D	7 2	0 0	N D.
6	Severe dysplasia	118	0 4	0 2	2 9	0 0	0 0

Filter pore size 5μm
Filtration rate 12 ml/min
Pressure time 60 seconds
Fixative MFA
N D = not determined

small to draw definitive conclusions, there was no obvious selective loss of abnormal cells on the filter or in the filtrate.

Calculation of Cell Recovery Rates

Most of the cell distribution proportions presented thus far were calculated by counting the total number of cells found on the slide, on the filter and in the filtrate. The possibility remained that cells were lost during other steps in the preparation procedure. In order to evaluate this possibility, a 90μl sample was taken from the 9-ml starting cell suspension to determine the number of cells. This sample was spread evenly over a glass slide, air dried, ethanol fixed and Papanicolaou stained. The entire slide was counted, in contrast to the sampling of 24 1-sq-mm fields, as described in "Materials and Methods." By multiplying the number found by 100, the number of cells originally present in the starting suspension was determined. This method was highly reproducible. A 2.4% coefficient of variation was found for repeated counts of the same sample, and a 6.2% coefficient of variation was found for counts done on multiple slides made from one sample. The remainder of the cell suspension was processed further as described in "Materials and Methods," and the total number of cells on the slide, on the filter and in the filtrate was calculated. Since most of the cells were found on the slide, the method by which this number was calculated was evaluated for five samples. The 24 1-sq-mm fields sampled in a regular pattern (taking care that they were evenly distributed over the slide) were counted on three different occasions, using a different pattern each time. None of these patterns produced a consistently higher or lower count. A 9.7% coefficient of variation was found.

Having determined the accuracy of both counting methods, the experiment was done with five different cervical samples; the results are shown in Table VI. As can be seen there, a higher number of cells was found for all five samples by adding the cells in the different fractions than was calculated for the starting cell suspension. This discrepancy may reflect some cell loss during the preparation of the "90μl samples," for instance, by adherence of some cells to the tip of the pipet. In any event, this comparison of cell recovery indicates that the pressure-fixation technique causes no greater cell loss than does a "standard" smear preparation method.

Reproducibility

To evaluate the reproducibility of the filter technique in routine use in our laboratory, slides and filters were evaluated on 16 more occasions over a one-month period. The conditions applied were 5μm-pore filters, 12 ml/min filtration rate, 60 seconds of pressure and MFA fixative. A mean cell density on the slide of 78/sq mm (S D. = 20; range = 43 to 111) was found whereas a mean of 1.2% (S.D. = 1.2%; range = 0 to 5%) of the cells remained on the filter.

The variability in cell distribution on the slides described in Tables II and IV (35 slides) was evaluated by calculating the coefficient of variation of the cell densities. The mean coefficient of variation was 50% (S D. = 12%; range = 24% to 72%) at a mean cell density of 82 cells/sq mm (S.D. = 10; range = 66 to 109).

Figure 2A shows a slide prepared by the pressure-fixation procedure. Note that the cell area is well marked, exactly matching the area on the filter where the cells were placed. Cell distributions are shown in Figures 2B for a normal sample and 2C for an abnor-

Table VI *Total Number of Epithelial Cells, Calculated According to Two Different Methods*

Sample no.	Cell number calculated	Cells observed in				
		Slide	Filter	Filtrate	Total	Recovery (%)
1	43,100	44,960	80	0	45,040	104
2	69,800	80,420	240	0	80,660	116
3	40,900	48,690	160	0	48,850	119
4	52,300	63,645	240	80	63,965	122
5	42,100	49,817	160	0	49,977	119

Filter pore size: 5μm.
Filtration rate: 27 ml/min.
Pressure time: 15 seconds.
Fixative: ethanol.

mal one, containing many leukocytes. The morphology of the cells, including the more fragile parabasal, columnar and abnormal ones, is well preserved. Figure 2D shows cells at higher magnification, including a parabasal cell and some columnar ones. Note that the cilia of one of the columnar cells are clearly visible.

Discussion

The present study further developed the "cytopress" slide preparation method originally proposed by Tolles and co-workers.[15] With their method, monodispersed cell suspensions were deposited on a polycarbonate (Nuclepore) filter, and the cells were then transferred to a glass slide by means of controlled pressure; the cells were subsequently fixed to the glass slides after removal of the pressure by placing slide and filter in fixative. The present modification of the original technique uses a sponge in place of a plastic sheet between the metal pressure block and the filter, allowing simultaneous pressure-transfer of the cells from filter to slide and fixation of the cells on the slide. The sponge is made of polyether, has a flat surface, is composed of relatively uniform, small holes and is quite flexible. A sufficient volume of fixative is easily taken up; after the pressure is applied, the sponge is flattened slightly; the flattening allows good contact of the sponge with the entire filter, evenly distributing the pressure as well as the fixative. Comparable results were obtained with other kinds of sponge material, e.g., latex rubber, which has the same physical qualities (results not shown). Although the sponge was used repeatedly for different specimens, no cross-contamination could be observed by examining a slide to which the freshly used sponge had been pressed. The sponge was used for several months (approximately 25 to 50 hours of exposure to the fixative), after which some deformation occurred.

This modified technique resulted in significantly higher cell concentrations on the slide than did the original method (Table I). The method appears relatively robust in that operating parameters that could

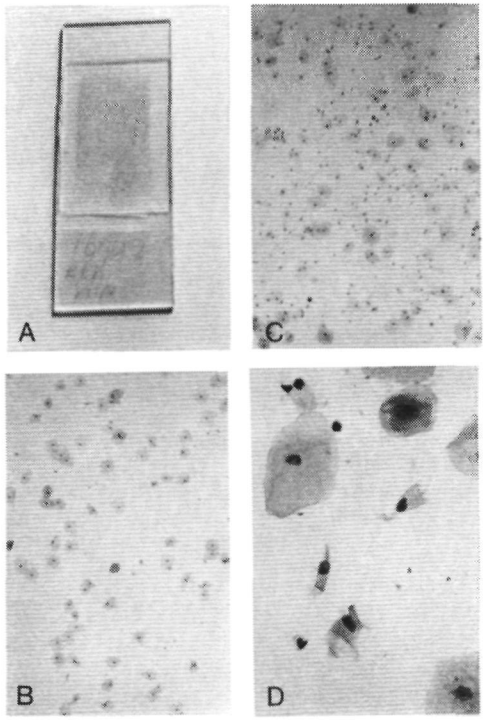

Figure 2
Cervical cytology slides prepared according to the pressure-fixation technique. (A) Slide showing the deposition area. (B) Area in normal specimen. (C) Area in abnormal specimen. (D) Higher magnification of area of slide shown in Figure 2B (Papanicolaou stain; B and C = × 40; D = × 250).

influence cell recovery, such as filtration rate and filter pore size during the initial deposition of the cells on the filter (Tables II and III) and the duration of pressure during the filter-to-slide transfer step (Table IV), can be varied over a reasonable range without significantly changing the final recovery of cells on the slide. In fact, cell recovery using the filter cytopress method appears to be high (Table VI), indicating that the method results in good adherence of the cells to the slide. Two fixatives, ethanol and a methanol-formalin-acetic acid mixture (MFA),[11] were used, both resulting in the same cell recovery on the slide (results not shown).

Far too few abnormal samples have been evaluated to allow definitive conclusions regarding recovery of abnormal cells (Table V). However, the preliminary data suggest that there is no selective loss of abnormal cells during the cytopress preparation procedure. The absence of carcinoma-*in-situ* specimens in the testing of cell recovery is of special concern since those cells are the smallest abnormal cells encountered in cervical cytology specimens. Those cells may be lost by passage through the filter holes. However, high rates of leukocyte recovery on the glass slides and the absence of leukocytes in the filtrate using the present procedure (data not shown) suggest that carcinoma-*in-situ* cells will not be lost selectively, at least on the basis of size alone.

Use of polycarbonate filters with 5μm or 8μm pores produced a selective loss into the filtrate of cellular debris and protein precipitates as compared with the 3μm filters (data not shown). The slides were thus significantly cleaner than routine cervical cytology specimens, resulting in a preparation that was easier to evaluate by manual techniques. Indeed, the morphology of some cells, such as endocervical columnar cells, was evaluated much more easily, because of both the good cellular dispersion and the cleanliness of the background.

In this study, cells preserved in PBS-20% ethanol were used, no studies have been done using fresh, unfixed cells. Studies are now under way to further evaluate this procedure on cells collected in a medium without preservatives as well as on other kinds of cytologic material.

The procedure described above is an easy and rapid method of preparing cytology slides from cells in suspension. Since the filter material is also available on rolls, the cell collection and transfer can be combined easily, making the development of a fully automated system feasible. At present, an apparatus is being evaluated that collects the cells at one position and, after moving the roll to a second position, transfers them onto a glass slide. A forthcoming paper will present the results achieved with this system.

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SECTION 2.4

Cytopress: automated slide preparation of cytologic material from suspension

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SUMMARY

In this paper a new automated system is introduced to prepare slides of cytological material from suspension. The system collects material on a filter tape by filtration and transfers it to glass slides by means of pressure-fixation. Using cervical cells as a model, results show that a well-defined cell number is evenly deposited over a standardized area, while a small number of cells is retained on the tape and a negligible number lost in the filtrate. Contamination is very small. Application of the system to other cytological material (fine needle aspirations, monolayer and cell suspension cultures, agar cultures and isolated nuclei) is shown. In general, more than one slide can be made from one sample. Several histological staining procedures as well as immunofluorescence labeling protocols can be applied to the preparations obtained in this way.

This system thus introduces a method that will standardize specimen preparation, is quick, saves operator time, and can be used for both diagnostic and research applications.

Light microscopical examination of cellular material by human observers has been performed almost exclusively on conventionally prepared and stained slides. Demands on the quality of the preparations were not so extreme, because the observer could correct for most preparative shortcomings. To improve this visual and qualitative evaluation, new techniques for cell interpretation are being developed. Our laboratory is involved in this research, developing, among others, specific immunohistochemical stains to detect the tissue origin of tumor cells (18-21), to detect quantitative morphologic and cytochemical changes using image analysis derived techniques (6,30,31) and to assess the malignant behaviour of tumor cells in an in vitro model system (5,8,9,27). These new techniques require the preparation of reproducible, well standardized specimens with homogeneously dispersed non overlapping singly lying cells or cell groups. Therefore, to complement this research, the development of new cytopreparatory techniques has begun in order to provide optimally prepared cytology slides (12,15,16). One of the steps in this specimen preparation procedure is the deposition of cells from suspension onto glass slides. Numerous approaches have been described to achieve this goal. These approaches can be subdivided into three classes: centrifugation (1,2,10,24-26), sedimentation (7,12,28) and transferring the cells through an intermediate filter onto a slide (2,11,16,17,22,23). In the last procedure the cells either remain on the filter (2,17) or are transferred to the slide by touch (22,23) or pressure (11,16). In a previous paper (16) we have evaluated the latter method. Cells were collected on a polycarbonate filter membrane and transferred to glass slides by simultaneous pressure-fixation. This procedure proved to be relatively robust with respect to parameters that might influence cell recovery such as filtration rate, filter pore size and pressure time. The present paper describes the use of an automated system based on the above described principle. Slides are prepared in an easy and quick way competitive with present conventional methods. They are suited for conventional cytology, fluorescence microscopy and quantitative cytology. Emphasis in this paper has been placed on the determination of cell recovery, distribution and

carry-over, using cervical cells as a model. Applications with other cytological and subcellular material are described.

MATERIALS AND METHODS

The Cytopress System

The principle of the system is shown in Figure 1. A roll of polycarbonate filter tape (designated B in Figure 1), 24 mm wide, maximal length 100 metres, with 5 µm diameter pores (Nuclepore, Pleasanton Ca, USA) is used to collect the cells from suspension and to transport them to a second position where they are transferred to glass slides. The filter tape is unrolled at one position (A) and rolled up at another (M). At the start of the procedure a funnel (E) is in position above the tape. Funnels with openings of different form and dimension in the bottom can be used. A fritted glass support (F) presses the tape to the funnel rim from below, resulting

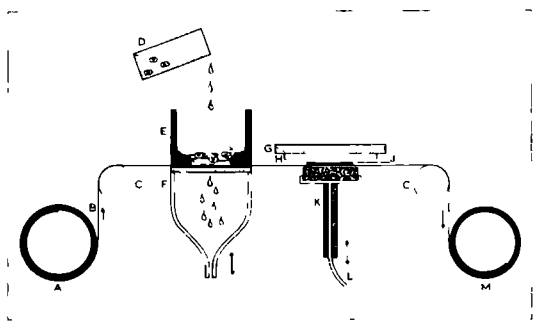


Figure 1. Schematic diagram of the "Cytopress" system.
A, roll from which the filter tape is supplied. B, polycarbonate filter tape. C, guiding roller. D, sample container with suspended cells. E, funnel to deposit cells on the tape. F, fritted glass base. G, perspex backing. H, glass slide. J, area with deposited cells. K, hollow pressure arm with a latex rubber sponge on top. L, tube, through which fixative is pumped to the sponge. M, take-up roll for used filter tape.

in a tight seal. The material in suspension (D) is poured into the funnel and a slight vacuum (-15 cm water pressure; minimal pressure -3,5 cm water, maximal -70 cm water) is applied underneath the filter. The suspension medium is then drawn through, leaving the cells on the tape. After the suspension has been aspirated the vacuum is disconnected, the funnel is moved away and the tape is transported to a second position. Here the material on the tape (J) is pressed against a glass slide (H) by a pressure arm (K). A latex rubber sponge moistened with ethanol fixative is mounted on top of this arm. The fixative is applied to the sponge by a tube (L), running through the arm. Through this pressure-fixation step the material is simultaneously transferred and fixed onto the slide. After the pressure application has been terminated the slide is removed from the device.

Figure 2a shows a front view and figure 2b a top view of the Cytopress system. Almost all handling has been automated. Before the procedure is started, both vacuum and time are set on the front panel (see Figure 2a). A numeric display in the center shows the chosen times. Vacuum time can also be regulated by hand using the central knob on this panel. A vacuum time of about 15 seconds and a pressure time of 20 seconds have been used in this study. Two different funnels have been used, one with a 16 x 35 mm retangular opening in the bottom, and one with a 10 mm diameter round hole. The choice of funnels is made by determining the number of cells one wants to inspect on the slide. For cervical cells, fine needle aspirations, cultured bladder tumor cells and tumor cell colonies the funnel with the rectangular opening is used. For cultured lung carcinoma cells and isolated nuclei the funnel with the round hole is chosen.

The first step in the operation is the simultaneous movement of the funnel and the positioning of a glass slide above the tape. Next, the glass support presses the tape against the funnel and the device waits for the operator to indicate that the sample has been poured into the funnel to start the vacuum. When the vacuum is terminated the glass support is lowered and the cells on the tape are transported to the "pressure position" (G in Figure 2b). Then the funnel is moved away from the tape. During this movement 80 µl of ethanol is pumped into the sponge by a peristaltic pump (P, figure

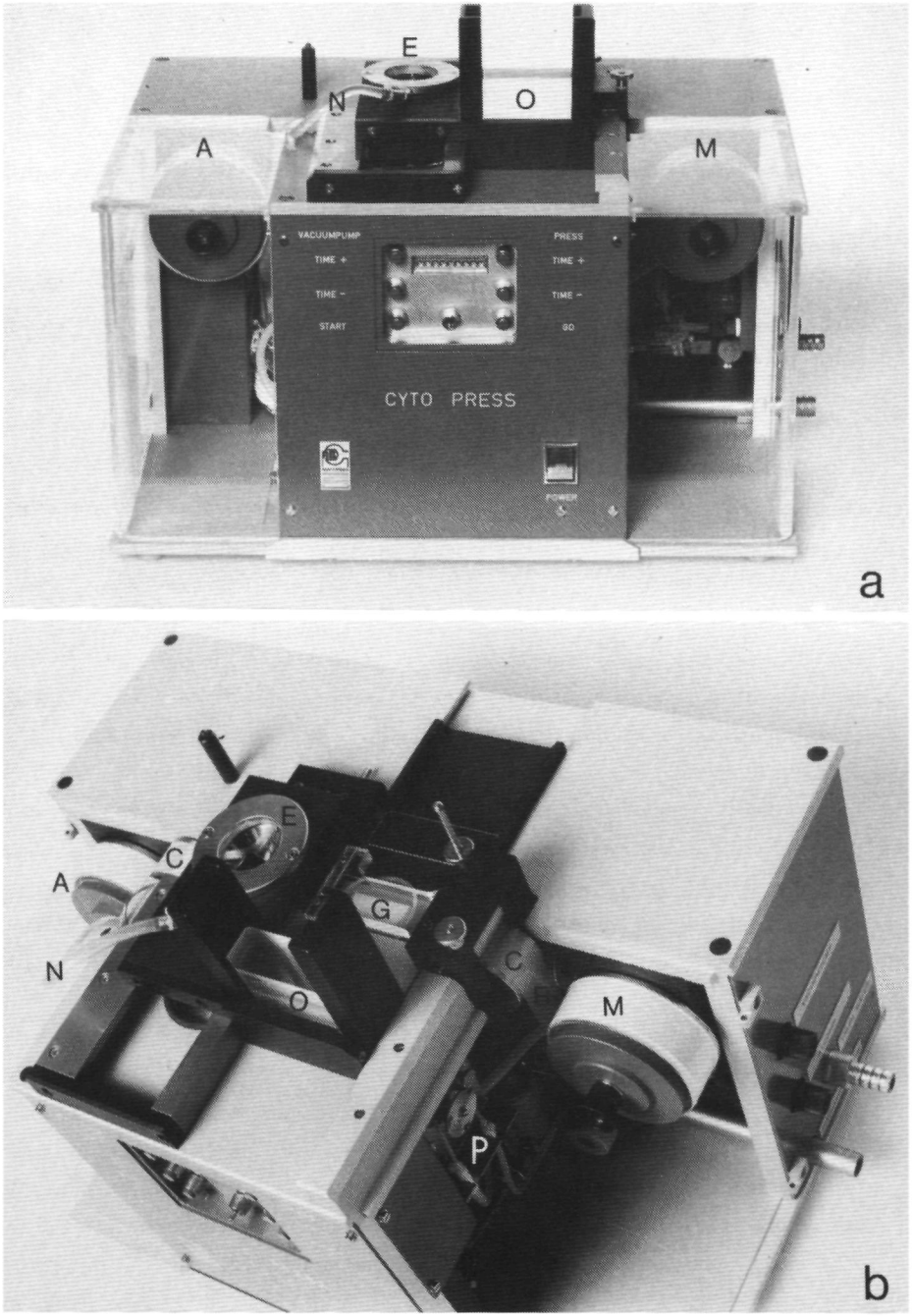


Figure 2. The Cytopress system.
a, front view; b, top view. For the explanation of the letters A-M see Figure 1. N, tube through which the tap water rinse flows to the funnel. O, stack of glass slides. P, peristaltic pump. For further details see the text.

2b), ensuring a constant amount of fixative for every sample. When the funnel is completely moved back it is rinsed with tap water (through the tube N), while the tape with the cells is pressed against the slide. After termination of the pressure the funnel is moved above the tape again. Concomitantly a metal plate pushes the bottom slide from the stack of blank slides (O) to a position above the tape and the slide with cells already transferred moves forward on a guide rail where it can be manually removed.

Clinical and biological material

Cervical cells.

Cervical cells were collected with a plastic spatula, suspended in phosphate buffered saline (PBS) containing 20% ethanol as a preservative (12) and stored at 4^{o}C. One to six days after collection the cells were disaggregated by hydrodynamic forces induced by a rotor device as described elsewhere (15). Cell concentration was determined using a Coulter Counter Model ZBl (Coulter Electronics, Hialeah, Florida USA) with a threshold setting that discriminated epithelial cells from most leukocytes present. Nine ml cell dilutions were made with PBS-20% ethanol containing approximately 36-72 x 10^{3} epithelial cells. On the average, four dilutions (range: one to 42) per sample could be made. The slides were post-fixed for 30 minutes in ethanol and then Papanicolaou (Pap) stained.

Fine needle aspirations.

Collection and processing of fine needle aspirations from the breast and the lung were performed in the same way as described for the cervical cells, but processed the same day. They were methanol post-fixed and stained for intermediate filaments (cytokeratin 18 or vimentin) using an immunohistochemical method (18,20,21).

Cell cultures.

Cells from a bladder carcinoma cell line (T24) were cultured as described elsewhere (29). For harvesting they were incubated briefly with trypsin (0,25%) - EDTA (0,1%), centrifuged (10 min x 400 g) and resuspended in PBS with 40% ethanol. After cell counting appropriate

cell dilutions were made in PBS-40% ethanol and processed on the Cytopress. The slides were post-fixed for 30 minutes in ethanol and Pap stained, or fixed in a methanol, formalin, acetic acid mixture (85:10:5 V/V/V) and stained quantitatively for DNA and protein using Feulgen-Pararosanilin(SO_2) Light Green (14).

Cells from a line of a small cell carcinoma of the lung were cultured as described elsewhere (3). These cells, growing as free floating aggregates, were centrifuged (5 min. x 500 g), resuspended in PBS and processed on the Cytopress. After post fixation in methanol (-20 °C, 1 min.) and acetone (three times 20 seconds) they were stained for vimentin (18).

Tumor cell colonies.

Material derived from a Grawitz tumor was cultured in double layer soft agar according to a method described by Hamburger and Salmon (4). After 28 days in culture the upper agar layer was carefully separated from the under layer. Two percent polyethylene glycol 1500 in 50% ethanol and PBS (1:1, V/V) was added to the dish and the separated layers were incubated for at least 24 hrs to soak off the colonies. The suspension was collected and the two layers were rinsed with extra volumes of the mixture described above. From these pooled suspenions, slides with cell colonies were prepared on the Cytopress. The slides were post-fixed for 5 minutes in methanol and Pap stained.

Endometrium tumor cell nuclei.

Endometrium tumor cell nuclei were obtained by thawing a liquid nitrogen stored sample (less than 0.2g) of endometrial tumor in PBS with 1 mM EDTA and 1 mM phenylmethylsulphonyl- fluoride. The material was kept at 4 °C and homogenized with 3 strokes of a tight fitting pestle in a Dounce homogenizer. The nuclear suspension was injected into the methanol, formalin, acetic acid fixative (see above), the nuclear concentration determined and approximately 40,000 nuclei introduced into the funnel with the round opening. To eliminate extranuclear material the nuclear suspension was rinsed several times in the funnel with extra volumes of fixative. The extranuclear material disappeared through the pores of the filter tape into the filtrate. Preparations were postfixed for an

additional 30 minutes in the same fixative mixture and stained quantitatively for DNA and protein using Feulgen-Pararosanilin(SO_2) Light Green (14).

Cell counting

The number of cervical epithelial cells found on the slide and the filter tape was calculated from counting 24 16X objective fields (covering approximately 22.5 mm^2). This counting was performed in 6 circular areas (3.5 mm diameter, 4 non overlapping 16X fields per circle) that were drawn in a regular pattern (see figure 3), using a standard template that exactly matched the cell area. To count the numbers of cells remaining on the filter tape, the part of the tape on which the cells were deposited was marked in advance and cut after the slide was made. It was attached to a glass slide and Pap stained.

In abnormal samples the abnormal cells and total epithelial cells (normals and abnormals) were counted. Abnormal cells were defined as cells consistent with a slight dysplasia or a more severe epithelial

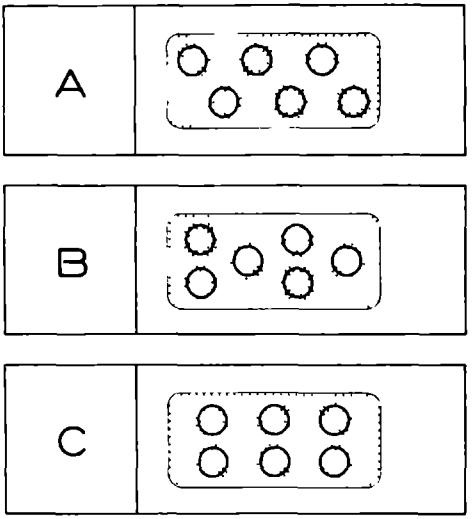


Figure 3. Schematic drawing of three slides with different counting patterns. Four non-overlapping 16x objective fields were counted in each circle. The pattern on slide A was used in this study.

abnormality. In cases in which the number of these cells was low (less than 100 per 24 fields) a 3x1 cm field (indicated by a second template) was counted. The number of abnormal cells expected in the entire (16x35 mm) cell area was then calculated.
The number of cells that passed through the filter tape was examined in pooled filtrates as described elsewhere (16).

RESULTS

Figure 4a shows two slides of cytologic samples that have been prepared on the Cytopress. Note that the borders of the areas are

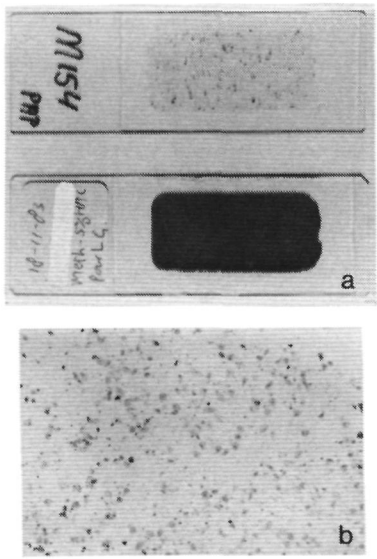


Figure 4. Cell distribution on "Cytopress slides".
a shows a macroscopic picture of two slides prepared using the funnel with the rectangular opening. The top slide shows a fine needle aspiration of the breast with the standard cell density. The lower one is a cervical specimen purposely overloaded with cells to demonstrate the sharply bordered cell area.
b shows a photomicrograph (x42) of an invasive carcinoma cervical specimen. a and b, Papanicolaou stain.

well defined. The areas exactly match that of the rectangle opening in the funnel used for the deposition of the cells on the tape. Figure 4b shows a microscopic picture at low magnification of a cervical specimen in order to illustrate the cell distribution patterns found in "Cytopress preparations".

Cervical samples were counted to compare the number of cells recovered on the slide, with those retained on the filter tape. First, the slide counting procedure itself was evaluated. Three different regular patterns, evenly distributed over the cell area (indicated on slide A, B and C in figure 3), were counted on three slides. The results in Table I show that none of these patterns resulted in a consistently higher or lower count. This suggests that the chosen procedure (pattern A in this study) gives a reasonable

Table I. Cervical cell number (epithelial cells/mm^2) on three "Cytopress" slides determined using three different counting patterns.

	Slide 1	Slide 2	Slide 3
Pattern A	60.1	59.4	73.2
Pattern B	55.8	49.6	59.5
Pattern C	50.1	54.3	62.3
Mean	55.3	54.5	65.0
Coefficient of variation	9.1%	9.0%	11.1%

estimate of the cell number for the entire specimen. In previous studies (15,16) this type of counting has proven to be highly reproducible. After this initial evaluation of counting patterns cervical samples were processed and a random selection, covering a broad range of cytologic diagnoses was evaluated during a nine month period. The total epithelial cell number and the number of abnormal epithelial cells found on the slide and the filter are shown in Table II. These numbers reflect the complete distribution of the suspended cells since previous studies (16) have shown that virtually all cells from suspension were recovered on the slide and the filter. From these counts, the percentage of cells retained on the filter has also been calculated. The total epithelial cell number found on the slides remains fairly constant for all cervical specimens. The number of cells found on the filter, however, rises for carcinoma in situ and particularly for invasive carcinoma specimens.

For the abnormal cell counts the same tendency for cell distribution between slide and filter is found as in the total epithelial cell number. However, for the abnormal cells the percentage of cells retained on the filter was less than for the total epithelial cell number. Cytologic evaluation of the abnormal cells on the filter did not show any specific preference of particular abnormal cell types for the filter.

The coefficient of variation (c.v.) of the cell counts of the 24 sampled fields per slide is a good measure of the cell distribution. Table III summarizes these c.v.'s and shows that the cell distribution becomes more uneven with increasing grade of abnormality. Part of this increase is caused by a large proportion of the cells being abnormals that are present in sheets or clusters.

During the same nine month period of Cytopress evaluation, pooled filtrates were analyzed for possible cell loss on eight different days. The results, summarized in Table IV, show that the loss of normal cells was negligible and that no abnormal cells were found in the filtrates.

Possible cross-contamination was studied in a series of seven normal cervical samples. After each processed sample a "blank" containing only PBS-20% ethanol was processed and the number of cells present on the resulting slides was counted. This solution, in which the cells were suspended after collection, is meant to act as a preservative to

Table II. Cervical cell number on slide and filter tape, found after using the Cytopress procedure. Total cell number is shown as epithelial cells/mm2, whereas the number of abnormal cells is shown for the entire slide or filter. The numbers are shown with standard deviation and range (in parentheses).

Cytological diagnosis	Number	Total epithelial cells/mm2			Total number of abnormal cells		
		Slide	Filter	% on filter	Slide	Filter	% on filter
Normal	10	88.9±21.7 (54.8-139.3)	8.9± 4.2 (3.7-18.4)	9.9± 2.7 (4.2-13.2)	-	-	-
Slight dysplasia	8	64.2±26.4 (35.5-111.7)	6.6± 2.8 (3.8-12.0)	9.9± 3.4 (4.3-14.9)	373±589 (100-1820)	5± 5 (0-16)	3.0± 3.0 (0-6.9)
Moderate dysplasia	6	53.8±22.4 (17.1-75.9)	4.2± 2.0 (1.8- 6.5)	7.5± 1.7 (4.5- 9.5)	1537±2091 (71-5530)	6± 3 (0-10)	2.1± 3.1 (0- 7.8)
Severe dysplasia	6	59.6±26.4 (32.0-94.4)	5.4± 4.0 (2.2-13.1)	8.1± 3.0 (4.7-12.2)	4483±6494 (101-15,307)	115±252 (2-630)	4.5± 2.8 (1.9- 9.4)
Carcinoma in situ	6	63.2±39.3 (28.8-130.3)	13.3± 7.5 (3.0- 22.7)	17.9± 6.7 (8.4-22.4)	10,129±10,470 (218-27,980)	531± 76 (3-1983)	5.6± 5.8 (0.3-15.2)
Invasive carcinoma	5	51.6±11.5 (32.1-61.5)	63.5± 81.7 (5.3-203.3)	39.5±27.5 (11.5-77.7)	11,362±7917 (358-19,367)	10,653±13,554 11-31,803)	29.2±26.9 (1.2-63.0)

Table III. Cell distribution (expressed as the coefficient of variation of the cell counting in each slide) in cervical slides, prepared on the Cytopress. The range is shown in parentheses.

Cytological diagnosis	Number	Coefficient of variation of number of cells per counted field
Normal	10	23.5 (16.5- 32.0)
Slight dysplasia	8	24.4 (18.1- 36.2)
Moderate dysplasia	6	39.4 (17.4- 76.6)
Severe dysplasia	6	32.1 (24.7- 39.4)
Carcinoma in situ	6	47.2 (28.4- 68.1)
Invasive carcinoma	5	69.9 (39.3-111.2)

Table IV. Total number of cervical cells found in pooled filtrates after using the Cytopress procedure. Filtrates were analyzed at 8 different days, during the 9 month Cytopress evaluation.

Day No.	Number of samples processed	Cytological diagnosis of the individual samples	Number of cells in filtrate	
			Normal	Abnormal
1	10	10 normals	150	-
2	5	4 normals	66	0
		1 moderate dysplasia		
3	8	2 slight dysplasias	153	0
		3 moderate dysplasias		
		2 severe dysplasias		
		1 carcinoma in situ		
4	5	1 normal	43	0
		1 slight dysplasia		
		1 severe dysplasia		
		1 carcinoma in situ		
		1 invasive carcinoma		
5	2	2 normals	10	-
6	3	2 normals	9	0
		1 slight dysplasia		
7	1	1 moderate dysplasia	1	0
8	1	1 invasive carcinoma	0	0

Table V. Determination of specimen carry-over in a series of seven normal cervical samples, determined after three different periods of sample storage. Numbers are shown as total number of cervical cells ± standard deviation, with the range in parentheses For further details see the text.

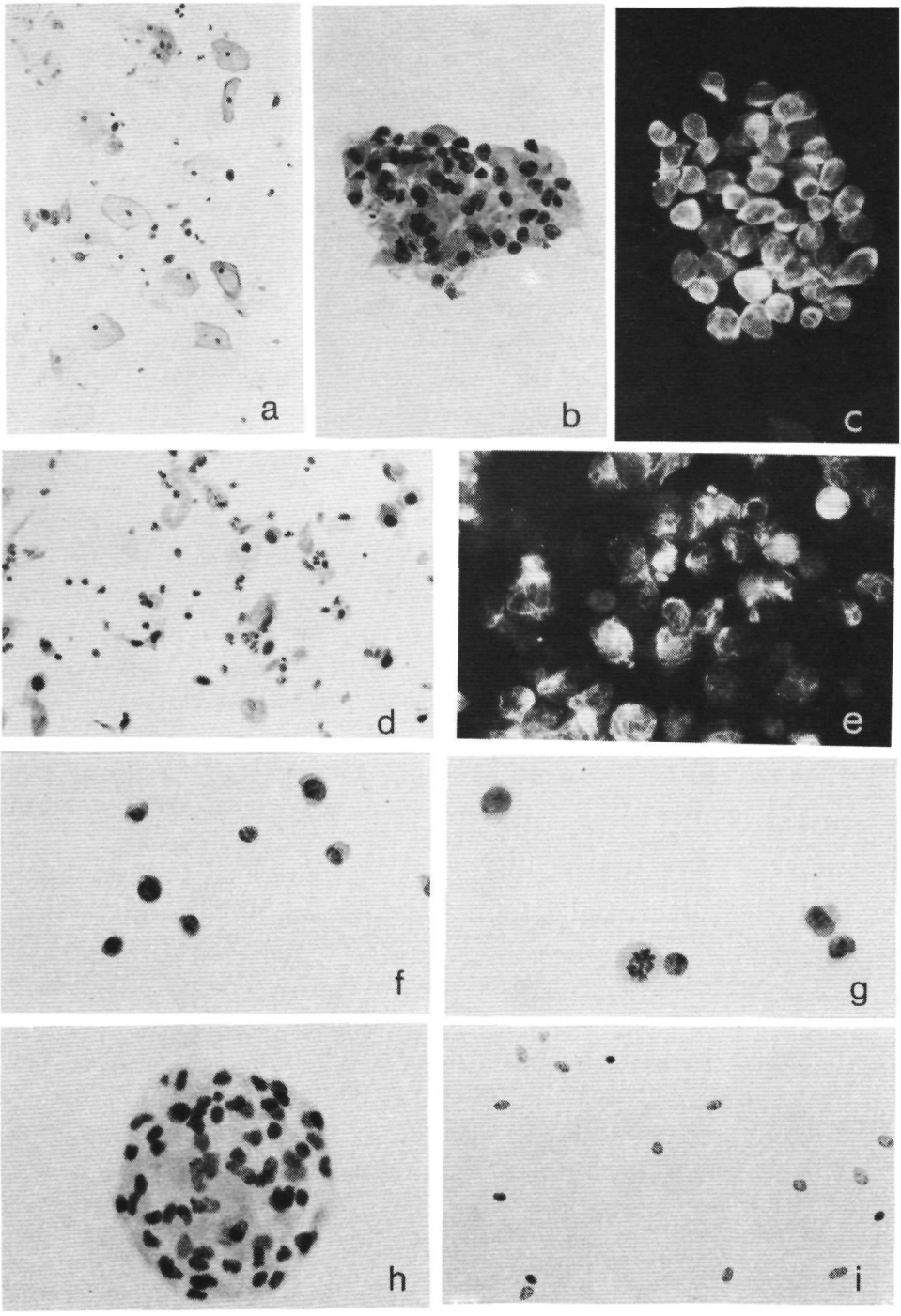
Days of storage	Number of cells carried over
1	10.4± 8.1 (1- 25)
3	5.1± 6.6 (2- 20)
6	8.5± 9.5 (1- 31)

allow storage of the cells for several days (12). Therefore, the counting was performed at three different storage times, with the same seven specimens. The results in Table V show that hardly any cells were carried over. These cells were mostly found at the edges of the slides.

Figure 5 shows examples of cellular material processed on the Cytopress. Figure 5a shows cervical cells of an invasive carcinoma specimen. Figures 5b and 5c show cells from fine needle aspirations of the breast. The cells in Figure 5b were Pap stained. Those in Figure 5c were stained for one of the intermediate filament proteins (cytokeratin 18), using an immunohistochemical method (20). Figure 5d shows Pap stained cells from a fine needle aspiration of the lung.

Cells from a small cell lung cancer cell line stained immunohistochemically for the intermediate filament protein vimentin are shown in Figure 5e. Figures 5f and g show cells from a bladder carcinoma cell line (T 24) that were Pap stained (5f) and quantitatively stained for DNA and protein (5g), using Feulgen-Pararosanilin(SO_2) Light Green (14). A mitotic figure is clearly seen in one of the central cells in figure 5g. A tumor cell colony of a Grawitz tumor grown in a double layer soft agar medium and Pap stained is shown in Figure 5h. Nuclei from an adenoacanthoma of the endometrium, quantitatively stained for DNA and protein, are shown in Figure 5i.

Figure 5. Examples of cells from slides prepared on the Cytopress. a, cervical cells from an invasive carcinoma sample (Thionine Feulgen-Congo Red (13), x170). b, cells from a fine needle aspiration of a large cell ductal carcinoma of the breast. (Papanicolaou stain, x170). c, cells of a fine needle aspiration of a ductal carcinoma of the breast, immunohistochemically stained for cytokeratin 18 (x375). d, cells of a fine needle aspiration of a squamous cell carcinoma of the lung. (Papanicolaou stain, x170). e, cells of a cell culture of a small cell carcinoma of the lung, immunohistochemically stained for vimentin (x280). f, cells of a monolayer cell culture of the bladder carcinoma cell line T24 (Papanicolaou stain, x270). g, The same cells as in f, now quantitatively stained for DNA and protein (Feulgen-Pararosanilin(SO_2) Light Green, x340). h, a tumor cell colony of a Grawitz tumor, grown in double layer soft agar. (Papanicolaou stain, x650). i, nuclei of an adenoacanthoma of the endometrium, quantitatively stained for DNA and protein (Feulgen-Pararosanilin (SO_2) Light Green, x270).


a
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i

The cytological specimen preparation procedure as outlined in this paper is a reliable method to deposit cellular material on glass slides. Cells or nuclei are deposited on a standardized area relatively easily and within a minimum of time. For most specimens in this study a filtering time of 15 seconds and a pressure time of 20 seconds have been used. These times were chosen as a result of experience with an earlier hand-based system (16). Together with various intermediate steps this results in a total processing time of 1 minute per specimen. Combined with a 15 second disaggregation time (15), cell counting and dilution, the total processing time from patient to slide can be kept within a few minutes.

The system, as it has been tested with cervical cells, results in slides with a well standardized cell number and with a minimum of cells remaining on the filter tape. A negligible number of cells is lost in the filtrate. Recovery rates from normal up to carcinoma in situ specimens are comparable to those found by Barrett and King (2) for collecting cells from body cavity fluids on Millipore filters. They are somewhat less then those found by Nielsen et al (11) for collecting cells from urine specimens on glass slides by a comparable Millipore filter imprint technique. In the latter studies however the slides had to be coated with gelatin-chrome alum. In the present study uncoated slides could be used, and precleaning of the slides was unnecessary. The higher number of cells retained on the tape for invasive carcinoma specimens could be caused by the more unfavourable conditions (more damaged cells caused by the neoplastic process, cell debris and blood cells present) the cells were exposed to, which made them more "sticky" to the filter. Still we feel that the number of cells, both total and abnormal, found on the slide is sufficient for a reliable diagnosis, especially since there was no preferential cell loss of abnormal cells. Part of the extracellular material such as protein and cell debris is found in the filtrate. Therefore the procedure results in cleaner slides than with conventional methods. The cell distribution is fairly homogeneous (see Figure 4b and Table III). Scarce numerical data on cell distributions with cell preparation procedures have been published so far. Watts and coworkers (28) have shown data for two cervical cell samples using

their sedimentation technique on polylysine coated slides. With the introduction of quantitative cell analysis and especially automated means of cell measurement (30), a well standardized cell distribution with almost equal cell densities per measured field is very important to make these measurements efficient. Cells in these slides can be more easily observed than in routine (cervical) slides because a monocellular layer is created with a majority of the cells and cell groups lying singly.

More than one slide per sample can be prepared in most cases, allowing several stains to be applied. For instance, stains for quantitative cell measurement (13,14) or immunofluorescent stains (18,20,21) can be used in addition to conventional cytology stains.

With respect to cross-contamination of cells we have found that per $36\text{-}72 \times 10^3$ normal cervical cells about five to ten cells can be carried over onto the next slide. When samples of cells of different origin are processed successively one should be aware of this possible contamination from the preceding preparation. However these cross-contaminated cells are found mostly at the edges of the slide, which makes recognizing them easy. A matter of concern might be the eventual cross-contamination of benign samples with malignant cells. Present studies are underway to investigate this possibility.

Fine needle aspirations are processed in the same way as cervical specimens and immunofluorescent labeling for intermediate filament proteins (Figure 5c) (18,20,21) has been applied. Together with conventional Pap staining this immunocytochemical method allows the determination of the tissue of origin of the tumor cells. For the latter procedure the samples must be processed on the day of collection.

"Cytopress preparations" can also be made from cultured cells. Cells cultured in monolayer (the bladder carcinoma cell line T24) or as free floating aggregates (a small cell carcinoma cell line) have been processed and Pap stained (Figure 5f), quantitatively stained for DNA-protein (Figure 5g) and stained for intermediate filament immunofluorescence (Figure 5e) For both fine needle aspirations and cell cultures the staining determines the medium in which the cells are suspended and processed in the Cytopress system. For morphological examination using the conventional Pap stain or for quantitative DNA protein staining, a PBS solution with 20 to 40%

ethanol concentration is needed to preserve optimal morphology. For immunofluorescent intermediate filament staining PBS without ethanol can be used.

New methods have been developed to assess the agressive behaviour and chemotherapeutic sensitivity of tumors, using a double layer soft agar system (4,5,8,9,27), in which single tumor cells are cultured in vitro. The evaluation of the morphology of the tumor cell colonies is difficult in the agar but with the Cytopress system it has been found that the evaluation of these colonies on slides is much easier. For more in depth studies in which isolated nuclei are measured to quantify DNA and nuclear protein, the Cytopress offers two advantages; slides are easily prepared and most extranuclear material is lost through the pores of the filters, thus resulting in cleaner slides. Unfixed nuclei in PBS can be processed but tests resulted in several cases in which nuclei were damaged or deformed. Fixing the cells in suspension resulted in nuclear preparations of excellent morphology, while no obvious change in recovery on the slide was noted.

In all cases an additional fixation of the cells or nuclei on the slides has been performed after Cytopress preparation. These fixations varied, depending on the staining to be applied. Studies are underway to determine whether the additional ethanol post-fixation for routine Pap staining can be omitted. In this way slides for routine cytology can be immediately stored for later staining.

Studies are underway to extend the capabilities of the Cytopress system to cytological samples that contain large amounts of extracellular material, such as pleural and ascitic fluids. Since this extracellular protein may possibly obstruct the pores in the filter techniques are being explored such as altering the suspension medium, changing vacuum pressure and time, and using alternative pore sizes.

In conclusion the described system is a highly useful device for the preparation of slides from a variety of cytological material. Because in most cases more slides per sample can be made, the diagnostic capabilities can be extended by using multiple stains including newly developed, more specific ones. For large scale processing the technique offers higher speed and easier automation

than other methods. In addition the quality of the specimen is superior for both visual and machine interpretation.

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CHAPTER III

STAINING

SECTION 3.1

Thionine-Feulgen Congo Red staining of cervical smears for the BioPEPR image analysis system

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Thionine-Feulgen-Congo Red Staining of Cervical Smears for the BioPEPR Image-Analysis System

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A staining method developed for use with BioPEPR (Biological Precision Encoding and Pattern Recognition), an automated image-analysis system for cervical smears, is described. The stain is a combination of the Feulgen procedure, with thionine-SO_2 as the Schiff reagent, and Congo red, which is used as a counterstain. The stain resulted in smears suitable both for microscopic diagnosis and for BioPEPR measurements made on photonegatives at a single wavelength of 545 nm. A high level of reproducibility and accuracy of nuclear and cytoplasmic area measurements was obtained. Nuclear integrated optical density could be well measured and was shown to be useful in discriminating between normal and abnormal cells. Using a combination of morphologic features, a high level of cell classification accuracy was reached. The possibility of using the stain for more detailed studies is discussed.

Cervical smears that are scanned by an automated image-analysis system are usually stained by the traditional Papanicolaou procedure. A major disadvantage of this five-component stain is the variation in staining between cells. Such cells will have different absorption characteristics, making it very difficult to extract morphologic parameters at a single wavelength; two[5,6] or even three[11] wavelength measurements seem to be required to give reliable results. Sophisticated and computation-intensive computer programs are then needed to compare the measurements from the different wavelengths.

In some cases, other staining procedures have been used. A combined fluorescent DNA-protein stain that makes it possible to measure nuclear and cytoplasmic parameters independently has been described.[3] Until now, this stain had been used exclusively to determine nuclear features.[1] In addition, other procedures have been reported in which only the nucleus is stained,[4,7] limiting measurements to nuclear morphology.

The BioPEPR image analysis system scans photonegatives made at a wavelength of 545 nm, using a special wide-field lens.[14] The lens is corrected for this wavelength and gives a 1μm resolution over an area of 8×8 mm. Smears stained according to the Papanicolaou procedure are very poorly suited for such a system due to the varying absorption characteristics of the cells at 545 nm.

This paper describes a new staining procedure that gives maximal contrast between nucleus, cytoplasm and background at the 545-nm wavelength. This makes it possible to obtain reliable measurements

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Table 1 *Thionine-Feulgen-Congo Red Staining Procedure*

Step	Solution	Time/handling
1	methanol-formaldehyde-acetic acid (85 10 5 v/v/v)	30 minutes
2	80% methanol	Rinse briefly
3	70% methanol	Rinse briefly
4.	50% methanol	Rinse briefly
5	deionized water	Rinse briefly
6	5-N HCl	60 minutes
7	4× deionized water	Rinse briefly
8	Thionine-SO_2 solution	60 minutes
9	3× SO_2 water	20 minutes total
10	2× deionized water	Rinse briefly
11	50% ethanol	Rinse briefly
12	70% ethanol	Rinse briefly
13	Congo red solution	15 minutes
14	3× 70% ethanol	10 minutes total
15	80% ethanol	Rinse briefly
16	96% ethanol	Rinse briefly
17	2× 100% ethanol	Rinse briefly
18	2× xylene	Rinse briefly

with a single wavelength. Moreover, the stain results in cells with blue nuclei and orange-red cytoplasms, making the smears well suited for light microscopic diagnosis.

Materials and Methods

Cervical samples were collected in suspension, disaggregated and monolayered onto a slide according to a procedure previously described.[8] The staining procedure, outlined in Table I, is performed at room temperature. The first step is the fixation. The "brief rinses" are done by dipping the slides 10 to 15 times in the appropriate solutions. The thionine-SO_2 solution is prepared according to van Duijn[13]; 0.5 gm of thionine is added to 250 ml of distilled water, and this solution is boiled for five minutes. After cooling to room temperature, enough distilled water is added to restore the volume to 250 ml. Next, 250 ml of melted tertiary butanol is added, followed by 75 ml of 1-N HCl and 5 gm of $Na_2S_2O_5$. After the flask is closed, the solution is stirred for 24 hours and then kept at 4 C for 48 hours. It is then ready for use. The solution can be kept for up to four weeks after preparation and must be filtered before use.

SO_2-water is prepared by adding 50 ml of 1-N HCl and 5 gm of $Na_2S_2O_5$ to 1 liter of deionized water.

Congo red solution is prepared by adding 4.42 gm of citric acid and 3.20 gm of Na_2HPO_4-$2H_2O$ to 300 ml of distilled water. This is stirred until the chemicals are dissolved, and then 700 ml of ethanol is added. Finally, 1 gm of Congo red dye is added, and the solution is stirred until all the dye is dissolved.

All solutions are freshly prepared for each batch of slides.

For BioPEPR measurements, the smears are photographed and scanned using a cathode ray tube scanner as described elsewhere.[14] Density profiles and area and nuclear optical density measurements are made with the "strobe mode," which measures transmittance at discrete points on the film. Effectively, these measurements are done using a spot with a 1μm diameter at 1μm intervals.

Manual area measurements are done by photographing the cells using a Zeiss Axiomat microscope (Carl Zeiss, Oberkochen, West Germany), printing the negatives (for a total magnification of 1,700×) and then weighing the nuclear and cytoplasmic components. This method gives a high level of reproducibility. A coefficient of variation of 8% was found for five repeated measurements of the relatively small nuclear area (18 sq μm) of a superficial cell. For the relatively scanty cytoplasm (150 sq μm) of a parabasal cell, a coefficient of variation of 3% was found.

Results

Absorption Characteristics

The absorption spectra of a thionine-Feulgen-stained nucleus and a Congo red-stained cytoplasm are shown in Figure 1. Other characteristics of the stain are given in Table II. These include the absorption maxima and the amount of absorption both stains have at 545 nm and at each other's maximum.

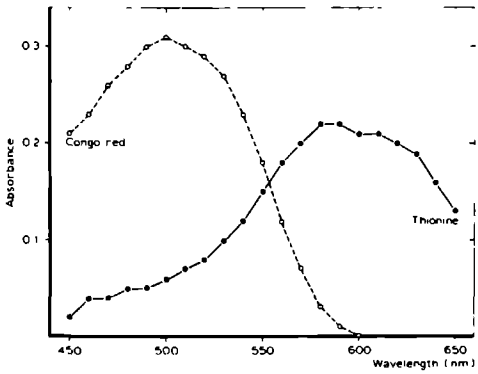

Figure 1
Absorption spectra of thionine and Congo red For the thionine spectrum, 4μm-diameter point measurements were done in the nucleus of a thionine-Feulgen-stained dysplastic cell For the Congo red spectrum, 20μm-diameter point measurements were done in the cytoplasm of a Congo red-stained superficial cell

Table II *Absorption Characteristics of Thionine and Congo Red*

	Absorption maximum	$\frac{A_{545}}{A_{max}}$	$\frac{A_{500}}{A_{585}}$	$\frac{A_{585}}{A_{500}}$
Thionine	585 nm	0.61	0.27	—
Congo red	500 nm	0.66	—	0.06

Morphologic Appearance

The stain resulted in cells with orange-red cytoplasms and blue nuclei, both of which may vary in intensity. The cytoplasms of superficial cells were faint, whereas cells originating from deeper layers of the cervix, as well as columnar, metaplastic and most abnormal cells, were more intensely stained. Most nuclei appeared blue; only those of superficial and most abnormal cells showed a dark blue to black appearance. Figure 2 shows three examples of cells stained with thionine-Feulgen-Congo red stain: a high intermediate cell (Figure 2A), a parabasal cell (Figure 2D) and a dysplastic cell (Figure 2G).

Area Measurements

Figures 2B, 2E and 2H show the respective density profiles in the horizontal direction, using the BioPEPR strobe mode, of the three cells depicted in Figures 2A, 2D and 2G. In all three cases, there was a

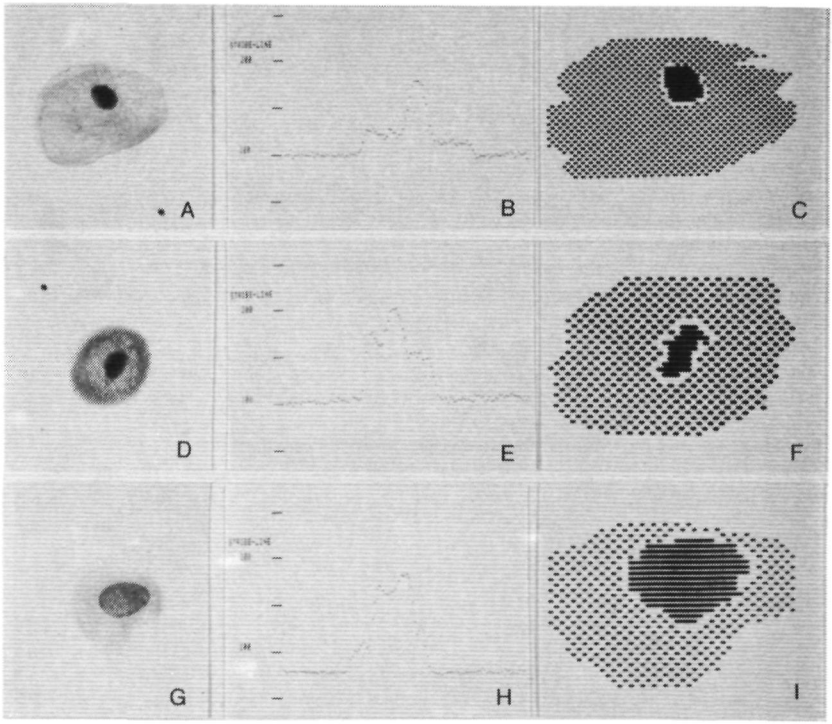

Figure 2
(A) High intermediate cell. (B) Horizontal density profile of the cell in Figure 2A. (C) BioPEPR representation of the cell in Figure 2A. (D) Parabasal cell. (E) Density profile of the cell in Figure 2D. (F) BioPEPR representation of the cell in Figure 2D. (G) Dysplastic cell. (H) Density profile of the cell in Figure 2G. (I) BioPEPR representation of the cell in Figure 2G.

Table III *Variability in Nuclear and Cytoplasmic Area Measurements on BioPEPR*

	Nuclear area		Cytoplasmic area	
	Mean (μm^2)	Coefficient of variation (%)	Mean (μm^2)	Coefficient of variation (%)
Dysplastic cell	162	9 0	572	3 0
High intermediate cell	80	3 0	1 688	5 5

steep transition from background to cytoplasm and from cytoplasm to nucleus These transitions, or inflection points, found in such profiles are used by the BioPEPR system to determine cytoplasmic and nuclear thresholds for area measurements. The nucleus can then be discriminated from the cytoplasm and the cytoplasm from the background. Figures 2C, 2F and 2I show the respective area measurements made by the BioPEPR system for the three cells.

Due to the high contrast, good reproducibility of the area measurements was obtained. Table III shows the reproducibility measurements of two different cervical cells that were automatically scanned by BioPEPR; the nuclear and cytoplasmic areas of these cells were scanned 200 times, and the means and coefficients of variation were calculated

The accuracy of the BioPEPR measurements was determined by comparing automatic with manual measurements. Figure 3 shows the results for such nuclear and cytoplasmic measurements of 20 intermediate cells selected from four different smears. The straight lines in Figures 3A and 3B represent the ideal situation, in which BioPEPR and manual measurements are equal. The correlation coefficients of these measurements are shown in Table IV; because a cervical smear consists of a variety of cell types, the correlation coefficients for a broader range of cells are also shown in the table.

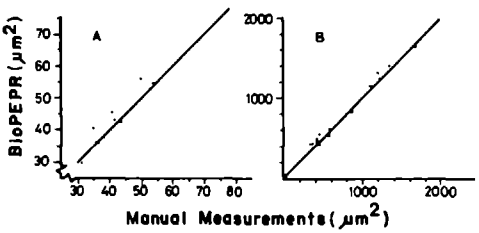

Figure 3
Comparison of BioPEPR and manual area measurements of (A) nuclei and (B) cytoplasms of 20 intermediate cells from four smears

Nuclear Integrated Optical Density Measurements

Figure 4A shows a nuclear integrated optical density (I.O.D.) histogram for intermediate cells from nine different smears. A coefficient of variation of 25% was found. If a correction factor is introduced to compensate for intersmear variations, a coefficient of variation of 21% is reached. This correction factor is found by taking the average nuclear I.O.D. of the intermediate cells within each smear and assigning to it a standard value, which is equal for all smears The number by which the measured average I.O D. has to be multiplied to get the standard value is the correction factor. In the BioPEPR system, 10 to 20 intermediate cells are measured from each smear in order to calculate this correction factor. The nuclear I O D. of other cell types is also multiplied by this correction factor since the variation among these cells has the same order of magnitude as that for the intermediate cells (results not shown). The histograms for corrected optical density for normal and abnormal cells are shown in Figures 4B and 4C, respectively.

Cell Classification

In the BioPEPR system, nuclear area, nuclear shape, nuclear I.O.D. and nuclear-cytoplasmic (N/C) area ratio measurements are made using the single wavelength approach, and cell classification is then implemented using a hierarchical decision tree strategy. This tree, which has been extensively discussed elsewhere,[14] classifies the cells as normal, unknown and abnormal. Table V shows these classifications for the different kinds of cells found in a cervical smear.

Discussion

The difficulty of using Papanicolaou-stained smears with the BioPEPR system led to the development of a new staining procedure The Feulgen technique is used for staining the DNA in the nucleus, with thionine-SO_2 as the Schiff reagent. Model experiments have shown that DNA is stoichiometrically stained in this way.[9] Fixation in the methanol-formaldehyde-acetic acid mixture gives reproducible DNA staining,

Table IV *Correlation Coefficients for Manual and BioPEPR Area Measurements*

	Nucleus	Cytoplasm
20 intermediate cells from 4 smears	0 86	0 97
35 different cells* from 4 smears	0 71	0 96

*6 superficial 8 intermediate 4 parabasal 7 columnar 4 metaplastic and 6 dysplastic cells

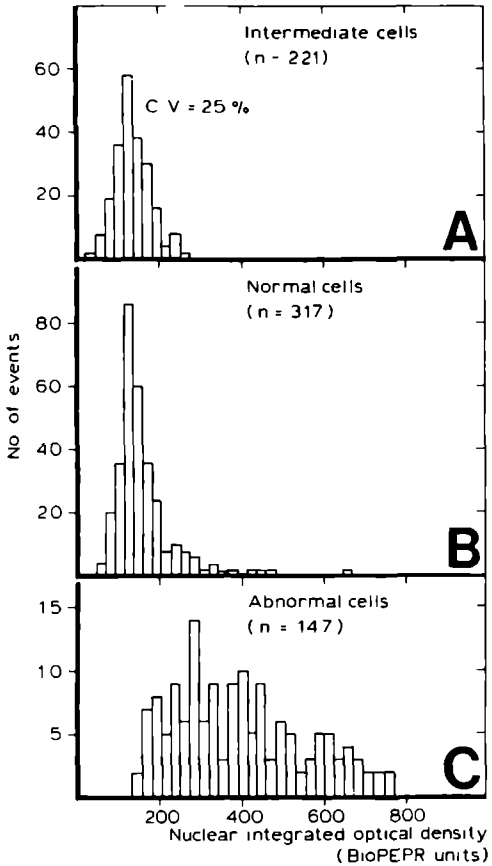

Figure 4
Nuclear integrated optical density histograms (A) Intermediate cells (from nine smears) (B) Normal cells (from nine smears) (C) Abnormal cells (from four smears)

which is rather insensitive to the small variations in HCl hydrolysis times [2] Congo red is used as a counterstain The staining intensity of Congo red can be changed by varying the acidity of the solution, increasing acidity results in a more intense Congo red staining After preliminary experiments, the best results were obtained using the procedure described in this paper The combination of the blue thionine and the orange-red Congo red resulted in stained smears that were very well suited for light microscopic diagnosis, this allows the same smear, after scanning by the BioPEPR system, to be used for screening by a cytotechnologist

Both stains have their absorption maxima separated by about 40 nm from 545 nm (Figure 1 and Table II), the wavelength at which the photonegatives are made However, at this wavelength they still absorb at about 60% of their maxima The combination of both stains at 545 nm gives a good dynamic range for optical density measurements, yielding a good separation between cytoplasm and background and between nucleus and cytoplasm for the different kinds of cells appearing in a cervical smear Due to this contrast, thresholding can easily be done, resulting in a high level of reproducibility and accuracy for the BioPEPR area measurements In the case of nuclear area measurements, a slight decrease in accuracy was found in going from intermediate to a broader range of cells This is mainly due to the parabasal and metaplastic cells The transition from the intensely stained cytoplasm to the nucleus is sometimes very gradual in these cells, resulting in an incorrect threshold, which gives in most cases an overestimated nuclear area

The variation found in the nuclear I O D measurements for intermediate cells is mainly due to small staining variations between smears and the way this density value is computed Although the staining conditions are kept as constant as possible, small staining variations are seen, even between smears from the same batch The nuclear I O D as measured by the BioPEPR system is a summation of the thionine and Congo red absorptions at 545 nm within the nuclear boundary With a constant amount of DNA present in the nucleus, variations in Congo red-stainable material above, within and under the nucleus will contribute to variations in the optical density In order to correct for the intersmear staining variation, a correction factor was introduced, resulting in a reasonable decrease of the coefficient of variation Coefficients of variation of 9%[10] and 13%[12] have been found for intermediate cells based on measurements of only nuclear stains

The small variations in nuclear I O D between the intermediate cells in smears is accompanied by a similar variation in the densities of the other cell types found in those smears This results in shifts of the total density histograms from smear to smear Using the correction factor this shift can be eliminated, and fixed thresholds can be set to discriminate normal from abnormal cells on the basis of nuclear I O D As shown in Figures 4B and 4C, a high level of discrimination can be obtained

With this single-wavelength approach and using the hierarchical tree decision strategy, a high level of

Table V *BioPEPR Classification of Singly Lying Cells*

	BioPEPR Classification							
	Normal		Unknown		Abnormal		Total	
Cell type	No.	%	No.	%	No.	%	No.	%
Superficial	262	96 3	10	3 7	0	0	272	100
Intermediate	334	90 5	33	9 0	2	0.5	369	100
Low intermediate	101	85 6	9	7 6	8	6 8	118	100
Parabasal	32	41 0	18	23 0	28	35 9	78	100
Columnar	21	55 3	8	21 1	9	23 7	38	100
Metaplastic	13	38 9	15	44 1	6	17 6	34	100
Slight dysplasia	11	27 5	7	17 5	22	55 0	40	100
Moderate dysplasia	15	20 3	14	18 9	45	60 8	74	100
Severe dysplasia	8	10 7	10	13 3	57	76 0	75	100
Carcinoma *in situ*	8	23 5	1	2 9	25	73 5	34	100

cell classification accuracy can be reached. Only parabasal and metaplastic cells are sometimes difficult to classify, due to the aforementioned problems in finding the correct threshold between nucleus and cytoplasm. In addition to the nuclear area, the nuclear I.O D. and N/C ratio are then overestimated. Results of a large-scale field test have shown that smears in which many of these cells are present (e.g., squamous metaplasia and postmenopausal atrophy) make up a great deal of the false alarms.[15] Present research is now focused on a better classification of these cells using cytoplasmic features such as area and shape.

An additional advantage of the stain is that smears can also be used for more extensive studies The fact that Congo red hardly absorbs at the absorption maximum of thionine (Table II) means that measurements made at this wavelength will be well suited for chromatin texture analysis and quantitative DNA measurements. An investigation is now being done on a microscope-based scanning system (Zeiss Axiomat) to evaluate this stain for these purposes.

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SECTION 3.2

The use of Light Green and Orange II as quantitative protein stains, and their combination with the Feulgen method for the simultaneous determination of protein and DNA

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The use of Light Green and Orange II as quantitative protein stains, and their combination with the Feulgen method for the simultaneous determination of protein and DNA *

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Summary. The protein dyes Light Green and Orange II were studied separately and in combination with the Feulgen-Pararosanilin(SO_2) and -Thionin(SO_2) method for the simultaneous determination of DNA and protein With polyacrylamide modelfilms the pH dependency specificity and stoichiometry of Light Green and Orange II have been investigated The results of both staining methods with different biological objects have been compared In addition the Feulgen-Thionin(SO_2) method was studied with model films with respect to its specificity and stoichiometry In biological objects it has been compared with the Feulgen-Pararosanilin(SO_2) method When combining the Light Green staining with the Feulgen-Pararosanilin(SO_2) procedure and the Orange II staining with Feulgen-Thionin-(SO_2), both Feulgen-DNA stainings, which were first applied, proved to be unaffected by the following protein staining procedure When the Feulgen procedure was carried out without the dye, followed by Light Green staining, the latter became reduced when a sulfite water rinse was included but was unaffected when a running tap water rinse was used In the case of the Orange II staining a serious reduction in dye binding capacity was found in both situations – When the Feulgen-Pararosanilin(SO_2) Light Green procedure was carried out on isolated nuclei with all dyes present, a decrease of protein dye binding was observed, similar to that found with the well-known Feulgen-Pararosanilin(SO_2) Naphthol Yellow S combination It is concluded that in spite of this reduction the latter two combinations can be used for the cytophotometric analysis of DNA and protein in the same object

Introduction

Various staining procedures have been developed for the combined cytophotometric analysis of DNA and protein in the same microscopical specimen (Van Duijn 1961 Mitchell 1967, Tas et al 1974 1978, Gaub et al 1975, Fukuda et al 1979 Mitchell et al 1981) All of these methods make use of the Feulgen procedure for the specific and quantitative staining of DNA For staining of proteins dyes are used which bind either electrostatically, like for instance Naphthol Yellow S (Deitch 1955, 1966, Tas et al 1974, 1978, Gaub et al 1975, Tas and James 1981), covalently, like for instance dinitrofluorobenzene (Mitchell 1967, Mitchell et al 1981) or covalently with the use of an intermediate such as ninhydrin (Fukuda et al 1979) or acrolein (Van Duijn 1961)

The acid dye Naphthol Yellow S, which can be very well combined with the Feulgen-Pararosanilin(SO_2) method has been most widely used for cytophotometric purposes so far (Deitch 1955, Tas et al 1974, Gaub et al 1975, Van der Ploeg et al 1979) This staining combination is however less suited for visual examination, due to the rather poor contrast of Naphthol Yellow S stained structures with light of intermediate wavelength Therefore in studies, such as in diagnostic pathology, in which a correlation is needed between a visual impression and cytometric data, a protein staining with higher visual contrast is desirable

As Naphthol Yellow S binds to the free basic side chains of a protein by its sulfonic acid group (Deitch 1955, Tas and James 1981), other dyes containing one or more sulfonic acid groups may be also used for protein staining Orange II and Light Green are comparable acid dyes containing one and three sulfonic acid groups respectively (Lillie 1969) Combination of Orange II with the blue staining Feulgen-Thionin(SO_2) for DNA (Van Duijn 1956), or Light Green combined with the magenta staining Feulgen-Pararosanilin(SO_2), both result in preparations with a well contrasting, differential staining of DNA and protein

In this paper several aspects of the Orange II, Light Green and Feulgen-Thionin(SO_2) staining as well as the combined Feulgen Thionin(SO_2) Orange II and Feulgen-Pararosanilin(SO_2) Light Green procedures are presented This study has been done using a polyacrylamide model system (Van Duijn and Van der Ploeg 1970) and in situ with various types of biological preparations

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Materials and methods

Polyacrylamide film experiments

Polyacrylamide gels were prepared according to Van Duijn and Van der Ploeg (1970) and further handled as described by Tas and Roozemond (1973) with the following modifications

Ammonium persulfate (3%) was used for polymerization Film punches (diameter 1 cm) have been used up to two weeks after preparation Staining and rinsing was performed at room temperature under continuous agitation on a Coulter Mixer (Coulter Electronics Limited, England) The measurements were performed in a Shimadzu digital double-beam spectrophotometer UV 150 (Shimadzu Seisakusho Limited Japan) with distilled water as measuring medium In advance the absorption maximum of the dye to be measured was determined, and measurements were performed at this wavelength

Experiments to estimate the amount of bound dye in relation to the amount of protein or DNA incorporated were performed as described by Tas et al (1978) For each concentration of protein or DNA wedge-shaped films were made of varying thickness up to 350 µm After staining and measuring the scanned area (diameter 5 mm) of each film was punched out and dried over night at 100° C After it was cooled to room temperature over $CaCl_2$ in a dessicator (during 3 4 h) it was weighed to obtain a measure for the thickness of the film and thus for the amount of substrate incorporated The method of the least squares was used to calculate the slope of the regression line per wedge-shaped film used In this way different slopes for different concentrations were obtained These slopes were used to plot the amount of bound dye against the concentration This concentration was expressed as the amount of substrate present in the solution that was mixed with the acrylamide solution before polymerization

For protein staining, concentrations of 0 1% of the different dyes were used dissolved in 1% acetic acid (pH = 2 8) Staining was performed for 30 min followed by rinsing in the same medium for 90 min with changes at 5 15 30 and 60 min In experiments in which the staining was performed at another pH, 1% acetic acid was adjusted to the appropriate pH with HCl or NaOH In these cases rinsing was performed in a solution with the same pH After staining and rinsing at the appropriate pH the films were finally rinsed twice in distilled water and measured spectrophotometrically

For DNA staining the model films were hydrolyzed for 30 min (or other times as indicated in the text) in 5 N HCl at room temperature After rinsing in 4 changes of distilled water they were stained in a Thionin(SO_2) solution as described by Van Duijn (1956) As a modification 30% tert-butanol was used instead of 50% tert butanol to avoid the strong shrinkage of the films, occurring in the latter medium The films were rinsed for 30 min in 4 changes of sulfite water (5 gm $Na_2S_2O_5$ 1 l distilled water 50 ml 1 N HCl) followed by two rinses in distilled water and measured afterwards

Experiments with biological material

Rat liver nuclei Rat liver nuclei were isolated as described by Fredericks and James (1978) The nuclear suspension (7 µl) was placed on glass slides then covered by a coverslip of 24 × 32 mm to induce an even distribution The coverslip was taken off the preparation dried in air for 5 min and then fixed for 1 h in a freshly prepared mixture of methanol, 40% formaldehyde and acetic acid (85 10 5, by volume) Thereafter they were rinsed in 3 changes of methanol, air-dried and kept at 4° C until staining

Chicken erythrocyte nuclei All handling was done at 4° C One ml fresh heparinized chicken blood was mixed with 9 ml of a solution containing 140 mM NaCl 8 mM Na_2HPO_4 $2H_2O$ 1 67 mM NaH_2PO_4 H_2O 50 units ml heparine (Organon Technika the Netherlands) pH = 7 4 (heparinized PBS) The cell suspension was centrifuged for 5 min at 350 × *g* The supernatant was discarded and the pellet resuspended in 8 ml heparinized PBS This was centrifuged for 10 min at 250 × *g*, the supernatant discarded and the pellet resuspended in 8 ml heparinized PBS One ml was taken out, centrifuged for 10 min at 250 *g*, the supernatant discarded and 1 ml 0 25 M sucrose, 1 mM Tris pH = 7 4 added After the pellet was resuspended, "7 µl preparations" were made and fixed in the same way as described for the rat liver nuclei The fixation step completely eliminated the cytoplasms

Pigeon erythrocyte nuclei Pigeon erythrocyte nuclei were prepared in exactly the same way as chicken erythrocyte nuclei

Human lymphocytes Human lymphocytes were isolated from healthy donors according to an elutriation technique (De Mulder et al 1981) The cells were centrifuged for 10 min at 250 × *g* and resuspended in the same PBS medium as described for the chicken erythrocyte nuclei without heparine The cell concentration was estimated using a Burker counting chamber and brought on 5×10^6 cells per ml

The cell suspension (30 µl) was brought on egg white-glycerin coated slides These slides were prepared by cleaning them overnight in xylene and placing them for 15 min in a solution of egg white glycerin (1 1 by volume) which had been diluted 1 100 with distilled water They were carefully taken out in a vertical position, the excess fluid, accumulated at the bottom of the slide was removed by touching it with filter paper The slides were dried in the vertical position for 30 min at 60° C Subsequently they were cooled to 4° C and the lymphocyte suspension brought upon

After the cells had settled for 30 min the slides were fixed in the same way as described for rat liver nuclei

Staining of protein and or DNA For protein staining the same dye solutions were used as described for the polyacrylamide film experiments The slides were briefly rinsed in distilled water and 1% acetic acid pH 2 8 or 4 0 (dependent on the pH at which the staining took place) Staining was performed for 15 min followed by a 20 min rinse in 1% acetic acid pH 2 8 (or 4 0) The rinsing solution was changed after 5 s 5 and 10 min Thereafter the slides were air dried rinsed in tert-butanol (three times) and xylene (three times) and mounted in Permount (Fisher Scientific Company USA) For DNA staining the slides were briefly rinsed in distilled water and hydrolyzed for 1 h in 5 N HCl at room temperature This was followed by a four times rinse in distilled water and staining either in Thionin(SO_2) (Van Duijn 1956) or Pararosanilin (SO_2) (Grauman 1953) Rinsing was performed during 20 min in sulfite water (changes at 1 and 10 min) or in running tap water as indicated in the text This was followed by a two times rinse in distilled water and air drying Then the preparations were dehydrated and mounted as described for the protein staining

Combined DNA-protein staining was performed by first applying the DNA-staining up to the rinsing in distilled water, followed by the protein staining procedure

Chemicals

The following dyes were used Orange II (lot Nr 011297 Aldrich Chemical Company USA), Light Green (lot Nr 88002 Koch Light Laboratories England), Naphthol Yellow S (lot Nr 379, Chroma, W Germany), Thionin (lot Nr 8570258 Merck, W Germany), Basic Fuchsin (Pararosanilin) (lot Nr 5426, Aldrich Chemical Company, USA)

The following biochemicals were used (all from Sigma Chemical Company, USA) bovine serum albumin (fraction V), histone (calf thymus, type II AS), pepsin (porcin stomach mucosa), deoxyribonucleic acid (calf thymus type I) ribonucleic acid (calf liver, type IV), glycogen (rabbit liver type X) Further heparin (Diosynth, the Netherlands) has been used All further chemicals were of analytical grade

Cytophotometry

Measurements were performed on an Axiomat scanning microscope (Zeiss Oberkochen W Germany) interfaced to an LSI 11 minicomputer (Digital Equipment Corporation USA) with RL 01 disc storage Half micrometer spot measurements were performed using a planapochromatic 100 × 1 30 objective at 0 5 µm distances between the successive steps and scanlines at a frequency of 50 measurements per second As a light source a Xenon arc (XBO 150W 1 Osram W Germany) was used while the illumination spot had a diameter of 10 µm in the object plane For determining absorption curves a monochromator was used with a constant bandwidth of 20 nm Other absorbance measurements were done using narrow band path interference filters For Napthol Yellow S stained preparations a 436 nm filter was used (65% transmittance at 436 nm 17 nm bandwidth at 50% of peak transmittance Zeiss W Germany) for Orange II a 484 9 nm filter (54% transmittance at 484 9 nm 19 7 nm bandwidth at 50% of peak transmittance) for Pararosanilin a 565 5 nm filter (55% transmittance at 565 5 nm 20 nm bandwidth at 50% of peak transmittance) for Thionin a 587 9 nm filter (51% transmittance at 587 9 nm 19 3 nm band width at 50% of peak transmittance) and for Light Green a 645 8 nm filter (58 5% transmittance at 645 8 nm 20 5 nm band width at 50% of peak transmittance) All these last filters were purchased from Schott W Germany

The transmittance values were immediately converted into absorbance values by the photomultiplier system and fed into the computer

For determining the amount of bound dye in the object interactively a window was placed around it taking into consideration that this was large enough to provide extra measuring points next to the object for later background determination A histogram was constructed of the measurements performed within this window It was characterized by a large group of low absorbance values constituting the background and showing a gaussian distribution A fit was done on these background points and the central value and standard deviation were determined

A contour was drawn around the object using as a minimum contour level the central value of the background plus three times the standard deviation All measurements within this contour were then corrected for the central background value and integrated resulting in the total absorbance of the scanned object

Objects that were both stained for DNA and protein were scanned twice using exactly the same window One scan was done at the wavelength for the DNA dye measurement and the second at the wavelength for the protein dye measurement Between these successive scans the light field diafragm had to be readjusted Then in each set of measurements the central value of the background was computed and the object contour determined in the image obtained at the wavelength for the DNA dye measurement This was done in a similar way as described for the single wavelength measurements This same contour was also used for the image obtained at the protein dye measurement Then in each set of measurements the absorbances within the contour were corrected for the central background values

As the absorbance spectra of the two chromophores show some overlap (see Fig 7) correction of the measured data falling within the contour in both images was necessary to get a true value for the amount of DNA and protein dye For this an approach was used as described by Van der Ploeg et al (1979) It is assumed that the resulting absorbance spectrum of the double stained preparation is the sum of the two separate spectra The true absorbance for each chromophore in each pixel of the object can then be calculated from the following two equations

$$T_1 = A_1 + bB_2 \quad (1)$$

$$T_2 = B_2 + aA_1 \quad (2)$$

T_1 is the total absorbance at wavelength 1
T_2 is the total absorbance at wavelength 2
A_1 is the absorbance of dye 1 at wavelength 1

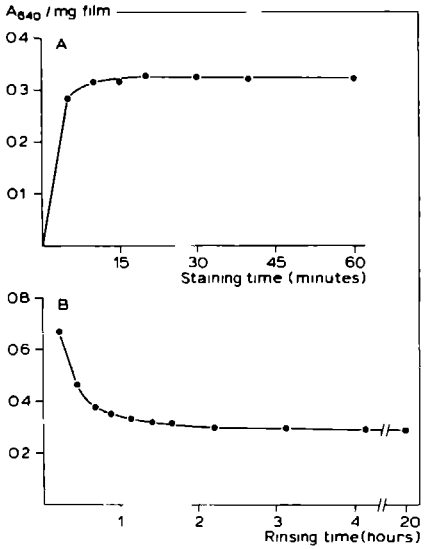

Fig 1 A Time course of Light Green staining at pH 2 8 of histone (1 mg/ml) containing polyacrylamide films **B** Time course of rinsing in 1% acetic acid pH 2 8 of albumin (5 mg ml) containing polyacrylamide films stained with Light Green pH 2 8

B_2 is the absorbance of dye 2 at wavelength 2
a is the percentage of A_1 measured at wavelength 2
b is the percentage of B_2 measured at wavelength 1
a and b can be computed from the individual spectra from single stained preparations and T_1 and T_2 can be measured

Therefore after rearrangement of (1) and (2) A_1 and B_2 can be computed as

$$A_1 = \frac{T_1 - bT_2}{1 - ab} \quad \text{and} \quad B_2 = \frac{T_2 - aT_1}{1 - ab}$$

The integrated absorbances of these corrected measurement were then determined within the contour

For the determination of the dye content per biological object the mean value was taken from 40 objects equally distributed over 2 slides

Results

Protein staining

Optimal staining conditions Optimal staining of protein incorporated in polyacrylamide films was achieved after 20 min As a typical example the course of the Light Green staining of histone is shown in Fig 1 A Similar results were obtained for the Light Green staining of albumin as well as for the Orange II staining of both proteins After 75 90 min all unbound dye was removed in all cases (see e g Fig 1 B) Therefore a staining period of 30 min and a rinsing time of 90 min was used as a rule in all further experiments For the Light Green and Orange II staining of biological objects e g chicken erythrocyte nuclei and

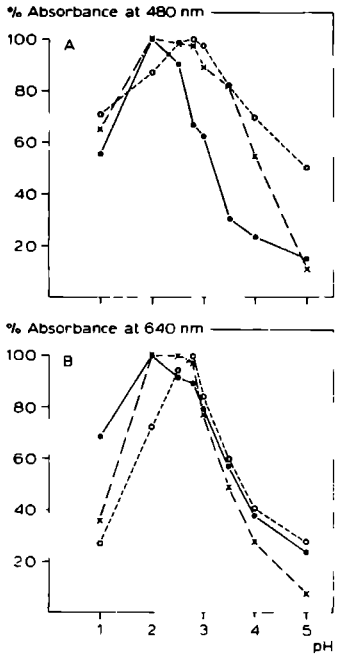

Fig 2A and B pH dependence of protein staining in polyacrylamide films A Orange II staining ● ● pepsin (75 mg ml) maximum absorbance mg film 0 128 × × albumin (50 mg ml) maximum absorbance mg film – 1 01 ○ ○ histone (20 mg ml) maximum absorbance mg film – 1 00 **B** Light Green staining ● ● pepsin (75 mg ml) maximum absorbance mg film = 0 860 × × albumin (10 mg ml) maximum absorbance mg film 0 390 ○ ○ histone (5 mg ml) maximum absorbance mg film – 0 655

rat liver nuclei, optimal staining was reached after 10 15 min, whereas the unbound dye was removed after 15 20 min A staining period of 15 min and a rinsing time of 20 min was used routinely therefore

The optimal pH of staining was evaluated in the polyacrylamide model system for three different kinds of proteins pepsin (a strong acid protein, pI below 1 0), albumin (a weakly acid protein pI 4 6) and the strong basic protein histone (pI 10 8) To obtain absorbances above 0 02 in the case of pepsin high concentrations had to be incorporated, whereas less albumin was necessary and far less histone As can be seen in the Figs 2A and B for both the Orange II and Light Green staining of pepsin the optimum is found near pH 2 0 for histone at 2 8 while for albumin it stretches from 2 0 to 2 8

Specificity The specificity of both protein dyes was tested under optimal conditions of pH (2 8) whereas Light Green also was tested under sub-optimal conditions (pH 4 0) The latter because Light Green staining in situ at optimal pH often results in very intensely stained objects which give rise to inaccurate cytophotometric absorbance readings

In addition to the three afore mentioned proteins other naturally occurring biological macromolecules such as DNA RNA glycogen and heparin were studied in the model system As can be seen in Fig 3 only proteins bind significant amounts of Light Green and Orange II The amount of dye binding of course was found to depend on the amount of basic amino acid residues present in the protein concerned (histone > albumin > pepsin)

Quantitation To investigate the quantitative binding aspects of both protein dyes films of different thickness containing variable amounts of protein were stained As a typical example Fig 4 shows the results of the stoichiometry experiment concerning the Orange II staining of albumin For all concentrations of albumin a linear relationship with a correlation coefficient of over 0 993 was obtained

In Fig 5A the slopes of these regression lines are plotted against the albumin concentration used and again a linear relationship has been obtained Similar experiments were

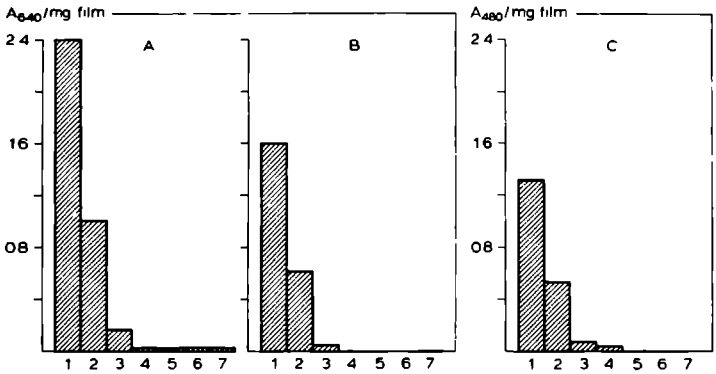

Fig. 3A–C Staining of different macromolecules in polyacrylamide films with Light Green at pH 2 8 (**A**) and pH 4 0 (**B**) and Orange II (**C**) 1 = histone 2 = albumin 3 = pepsin 4 = DNA 5 = RNA 6 = glycogen 7 = heparin concentration 10 mg ml in all cases

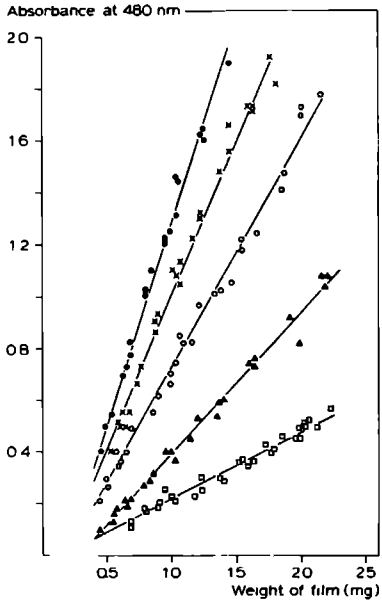

Fig 4 Orange II staining of albumin containing wedge shaped films ● = 25 mg/ml × = 20 mg/ml ○ = 15 mg ml ▲ = 10 mg ml □ = 5 mg ml The concentrations are expressed as the amount of albumin present in the solution, that was mixed with the acrylamide solution before polymerization

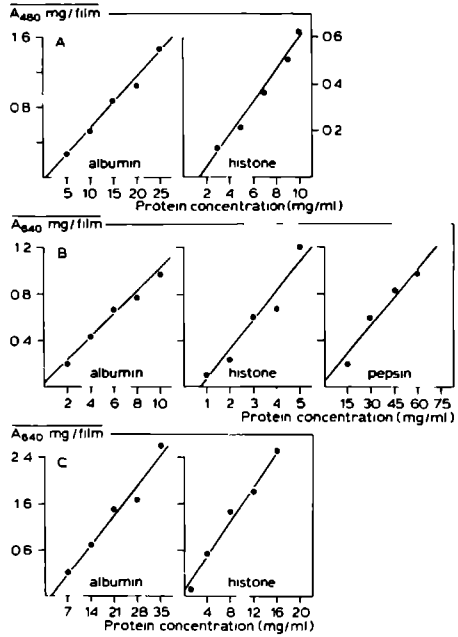

Fig 5 The stoichiometry of the staining of different proteins, studied with wedge-shaped films A = Orange II staining, B = Light Green staining at pH 2 8, C – Light Green staining at pH 4 0

performed for the Orange II staining of histone, and the Light Green staining of albumin, histone and pepsin

In all these cases a linear relationship was found (Figs 5A and B) The stoichiometry of the Light Green staining of albumin and histone was also studied at pH 4 0 The ultimate results from these experiments are shown in Fig 5C

Various biological objects were stained with Orange II and Light Green at pH 2 8 For a direct comparison between the two stainings the data are normalized by deviding the measured values by that measured for chicken erythrocyte nuclei in the same experiment (Table 1)

DNA-staining

In contrast to the numerous fundamental (model film) studies on the Feulgen-Pararosanilin(SO_2) method (see e g Hardonk and Van Duijn 1964, Duijndam and Van Duijn 1975a, b), fundamental information about the qualitative and quantitative aspects of the Feulgen-Thionin(SO_2) procedure still is lacking In view of the present study, therefore, some additional fundamental studies on the Feulgen-Thionin(SO_2) method were performed

Optimal staining conditions for Feulgen-Thionin(SO_2) To evaluate the optimal time for acid hydrolysis, DNA containing films were incubated for different periods of time in 5 N HCl at room temperature Optimal staining was achieved after 15 to 45 min of hydrolysis A hydrolysis time of 30 min was used in further experiments For chicken erythrocyte nuclei and rat liver nuclei the period of optimal hydrolysis was shifted to around 60 min

With regards to specificity, the Feulgen-Thionin(SO_2) staining did not prove to stain any RNA, glycogen, heparin, histone, albumin or pepsin in the model films (10 mg/ml concentrations tested)

Quantitation of the Feulgen-Thionin(SO_2) method To test the quantitative nature of the staining, experiments with wedge shaped model films were performed in the same way as described for the protein dyes Figure 6 shows that as a final result, a linear relationship is found

When applied to chicken erythrocyte nuclei and rat liver nuclei staining results quite comparable to the Feulgen-Pararosanilin(SO_2) method were achieved (Table 2)

Combined DNA-protein staining

Absorbance characteristics of Thionin and Orange II and of Pararosanilin and Light Green are shown in Fig 7 For both combinations the absorption maxima are well separated, with a small overlap at the respective peak wavelengths

Table 1. Dye content of different biological objects stained with Light Green (pH 2.8) or Orange II. The data are shown directly in arbitrary units (A.U.) as well in relation to the data measured for chicken erythrocyte nuclei (normalized)

Biological object	Light Green		Orange II	
	A.U. ± SD	Normalized ± SD	A.U. ± SD	Normalized ± SD
Chicken erythrocyte nucleus	20.16 ± 1.73	1.00 ± 0.08	8.66 ± 0.93	1.00 ± 0.11
Pigeon erythrocyte nucleus	24.37 ± 3.10	1.21 ± 0.15	10.67 ± 2.06	1.23 ± 0.24
Rat liver cell nucleus, 2 N	102.90 ± 13.01	5.10 ± 0.64	38.85 ± 4.24	4.48 ± 0.49
Human lymphocyte	143.07 ± 24.65	7.10 ± 1.22	58.59 ± 12.46	6.79 ± 1.44
Rat liver cell nucleus, 4 N	216.60 ± 20.08	10.74 ± 1.00	82.17 ± 11.07	9.48 ± 1.28

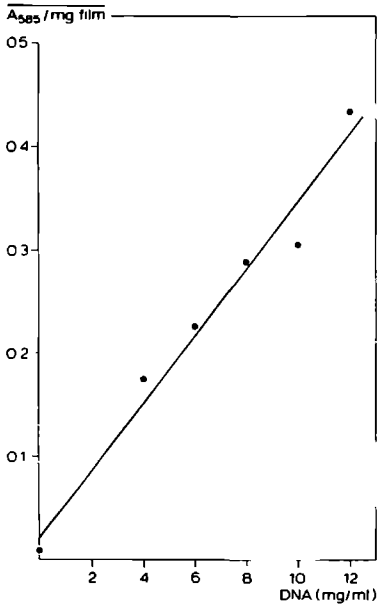

Fig. 6. The stoichiometry of the Feulgen Thionin(SO_2) staining of DNA, studied with wedge-shaped films

Table 2. Feulgen-Thionin(SO_2) and Feulgen-Pararosanilin(SO_2) DNA content of different nuclei expressed in arbitrary units ± standard deviation. In both Feulgen procedures a sulfite water rinse was included

	Feulgen Thionin-(SO_2)	Feulgen-Pararosanilin-(SO_2)
Chicken erythrocyte nucleus	21.81 ± 0.66	25.02 ± 0.65
Rat liver cell nucleus, 2 N	52.21 ± 1.57	60.46 ± 1.59
Rat liver cell nucleus, 4 N	108.51 ± 3.75	122.50 ± 4.96

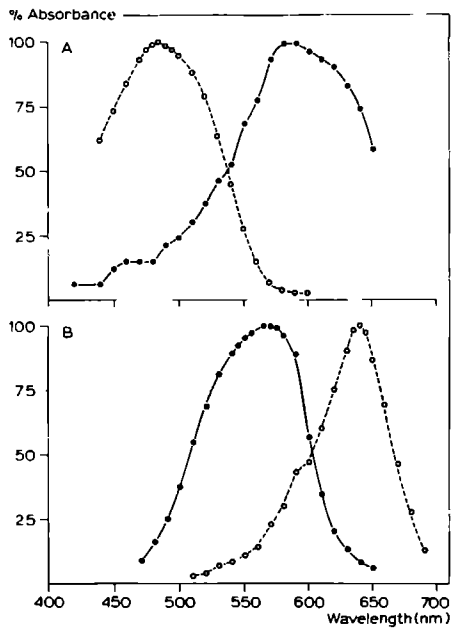

Fig. 7. A Absorbance curves of Orange II (o---o) and Thionin (●—●) 2 µm spot measurements were performed in Orange II or Feulgen-Thionin(SO_2) stained rat liver nuclei. The maximum absorbance of Orange II found at 485 nm, was 1.30. The maximum absorbance for Thionin, found at 585 nm was 0.32. B Absorbance curves of Light Green (o---o) and Pararosanilin (●—●) 2 µm spot measurements were performed in Light Green or Feulgen-Pararosanilin(SO_2) stained rat liver nuclei. The maximum absorbance of Light Green, found at 640 nm, was 0.41. The maximum absorbance of Pararosanilin, found at 565 nm, was 1.18

When DNA and protein staining is performed subsequently, mutual influence may occur resulting in the fact that the amount of dye bound differs from the amount bound in single stained preparations. Therefore in a first attempt this phenomenon was studied by imitating the

other staining simply by applying exactly the same staining conditions but with omitting the dye in the staining solution

Influence of protein staining on DNA staining Table 3 shows the influence of the Light Green staining conditions (pH 2 8 and 4 0) on the dye binding with the Feulgen Pararosanilin-(SO_2) method and Table 4 the influence of the Orange II staining conditions (pH 2 8) on the dye binding with the Feulgen-Thionin(SO_2) method As can be concluded from these results, the Light Green post staining conditions have no clear influence on the Feulgen-Pararosanilin(SO_2) data, while a slight decrease of the Feulgen-Thionin(SO_2) results is found after a subsequent Orange II "treatment"

Table 3 Influence of the Light Green staining conditions on the Feulgen Pararosanilin(SO_2) staining In the Feulgen Pararosanilin-(SO_2) staining a sulfite water rinse was included Amounts of dye bound are shown in arbitrary units ± standard deviation

Type of nucleus	Feulgen Pararosanilin (SO_2)	Feulgen Pararosanilin-(SO_2) followed by Light Green pH 2 8	Feulgen-Pararosanilin (SO_2) followed by Light Green pH 4 0
Chicken erythrocyte nucleus	23 43 ± 1 27	21 74 ± 0 99	21 26 ± 1 72
Rat liver cell nucleus 2 N	60 76 ± 4 57	61 48 ± 3 34	59 71 ± 2 15
Rat liver cell nucleus 4 N	122 50 ± 4 96	123 20 ± 6 12	119 16 ± 6 22

Table 4 Influence of the Orange II staining conditions on the Feulgen Thionin(SO_2) staining In the Feulgen Thionin(SO_2) staining a sulfite water rinse was included Amounts of dye bound are shown in arbitrary units ± standard deviation

Type of nucleus	Feulgen Thionin (SO_2)	Feulgen Thionin (SO_2) followed by Orange II
Chicken erythrocyte nucleus	21 92 ± 1 90	18 64 ± 1 19
Rat liver cell nucleus 2 N	49 98 ± 3 86	46 47 ± 3 01
Rat liver cell nucleus 4 N	102 97 ± 4 94	96 62 ± 5 47

Influence of DNA staining on protein staining The influence of DNA staining conditions on the Light Green staining is shown in Table 5 The Light Green staining was performed at pH 2 8 for chicken erythrocyte nuclei and at pH 4 0 for chicken erythrocyte nuclei and rat liver nuclei The preceding DNA staining without dye was done in two different ways One in which a sulfite water rinse was included and one with a running tap water rinse As can be seen in all situations when a sulfite water rinse was included a reduced Light Green staining was observed, whereas when running tap water was used no significant difference could be noticed In the case of Orange II staining (Table 6) a reduced protein staining is found after a simulated DNA staining including both the sulfite water and the running tap water rinse In the latter case the reduction is less but still unacceptable This makes the combination of Feulgen Thionin(SO_2) with Orange II unsuitable for quantitative protein measurements

Simultaneous cytophotometric determination of DNA and protein As already mentioned (Fig 7) some spectral overlap between Pararosanilin and Light Green occurs The relative absorbance of Pararosanilin at 645 5 nm the wavelength at which Light Green is measured, has been determined by 1 µm spot measurements in areas of different intensities in Feulgen-Pararosanilin(SO_2) stained rat liver nuclei A mean of 4% (standard deviation = 0 4) was found In a similar way the relative percentage of Light Green absorbance was determined at 565 5 nm, which showed a mean of 20% with standard deviation = 2 0 Using these two 'correction factors' the corrected values for Pararosanilin and Light Green absorbances were computed in double stained preparations and compared with those found in single stained ones (Table 7)

As can be seen similar Feulgen-Pararosanilin(SO_2) values were measured Quite different data were obtained with the protein staining The Light Green values in the double stained preparations were considerably lowered dependent on the type of nucleus For chicken erythrocyte nuclei a reduction of about 50% was found (both at pH 2 8 and 4 0) and for rat liver nuclei a reduction of about 16%

Table 5 The influence of the Feulgen Pararosanilin(SO_2) (F) staining conditions on the Light Green staining (LG) Amounts of dye bound are shown in arbitrary units ± standard deviation

Type of nucleus	LG, pH 2 8	LG pH 2 8 preceded by F with a sulfite water rinse	LG, pH 2 8 preceded by F with a running tap water rinse	LG pH 4 0	LG pH 4 0 preceded by F with a sulfite water rinse	LG pH 4 0 preceded by F with a running tap water rinse
Chicken erythrocyte nucleus	18 75 ± 1 60	13 59 ± 1 88	20 96 ± 1 68	8 50 ± 1 13	5 65 ± 1 10	8 69 ± 1 38
Rat liver cell nucleus, 2 N	–		–	69 30 ± 9 59	58 09 ± 5 62	73 68 ± 11 34
Rat liver cell nucleus 4 N		–	–	134 62 ± 17 56	115 50 ± 14 72	132 32 ± 13 10

Table 6 The influence of the Feulgen Thionin(SO_2) (F) staining conditions on the Orange II binding Amounts of dye bound are shown in arbitrary units ± standard deviation

Type of nucleus	Orange II	Orange II preceded by F with a sulfite water rinse	Orange II preceded by F with a running tap water rinse
Chicken erythrocyte nucleus	12 11 ± 1 28	5 48 ± 0 69	7 24 ± 0 83
Rat liver cell nucleus, 2 N	64 12 ± 3 74	36 39 ± 4 26	47 95 ± 6 20
Rat liver cell nucleus 4 N	133 46 ± 9 02	69 45 + 9 03	100 57 + 8 10

Table 7 The Feulgen Pararosanilin(SO_2) (F) DNA and Light Green (LG) protein content of isolated nuclei determined using the individual and combined stainings In the Feulgen Pararosanilin(SO_2) staining a running tap water rinse was included Amounts of dye bound are shown in arbitrary units ± standard deviation

Staining	Chicken erythrocyte nucleus	Rat liver cell nucleus	
		2 N	4 N
F	23 92 ± 0 79		–
F in F-LG, pH 2 8	21 83 ± 1 05	–	–
LG, pH 2 8	18 02 ± 1 86	–	–
LG in F LG, pH 2 8	9 50 ± 1 45		–
F	26 20 ± 1 40	60 24 ± 2 45	123 23 ± 5 80
F in F LG, pH 4 0	24 25 ± 1 32	61 18 ± 2 17	122 12 ± 6 27
LG pH 4 0	8 25 ± 1 26	69 30 ± 9 59	134 62 ± 17 56
LG in F-LG, pH 4 0	4 07 ± 0 54	57 45 ± 8 32	114 39 ± 16 85

Table 8 The Feulgen Pararosanilin(SO_2) (F) DNA and Naphthol Yellow S (NYS) protein content of isolated nuclei determined using the individual and combined stainings In the Feulgen Pararosanilin(SO_2) staining a running tap water rinse was included Amounts of dye bound are shown in arbitrary units ± standard deviation

Staining	Chicken erythrocyte nucleus	Rat liver cell nucleus	
		2 N	4 N
F	26 02 ± 1 55	64 62 ± 3 51	132 19 ± 6 12
F in F-NYS	24 06 ± 1 20	65 10 ± 3 08	129 10 ± 5 08
NYS	5 06 ± 0 74	22 02 ± 2 88	42 38 ± 6 30
NYS in F NYS	3 27 ± 0 56	17 24 ± 4 19	32 49 ± 4 94

For comparison the well-known Feulgen Pararosanilin-(SO_2) Naphthol Yellow S method (Tas et al 1974) was also applied on the same objects The percentage of Naphthol Yellow S absorbance at 565 5 nm was 1% (standard deviation = 1 0) Pararosanilin absorbs for 6% (standard deviation = 0 4) at 436 nm These correction factors were used and the results are shown in Table 8 No significant differences were found in the Feulgen Pararosanilin(SO_2) staining but definite reductions were observed in the Naphthol Yellow S staining of chicken erythrocyte nuclei (40%) and rat liver nuclei (20%)

Discussion

In this paper the possibility was investigated of combining the Feulgen method with protein stains for the simultaneous quantitative determination of DNA and protein

For DNA staining, the quantitative nature of dye binding with the conventional Feulgen technique using Pararosanilin(SO_2) as the Schiff reagent, is sufficiently known With regards to Feulgen-Thionin(SO_2) there is less experience, although some general aspects have been adequately described (Van Duijn 1956) This staining procedure proved to be also specific and quantitative reliable (Fig 6 Table 2) for DNA

In the case of protein staining, Orange II and Light Green are dyes which enable specific (Fig 3) and quantitative reliable (Fig 5) staining Both dyes have a pH optimum ranging from 2 0 to 2 8 (Fig 2) which is in good agreement with the data published for Naphthol Yellow S (Tas et al 1974, 1978)

At pH 2 8 a constant stoichiometric relationship between the amount of protein and the amount of bound dye is found (Fig 5) When staining different biological objects similar results are found for both dyes (Table 1) Light Green binds to protein also under the non-optimal condition at pH 4 0 in a specific (Fig 3 B) and quantitative reliable way (Fig 5), which is again similar to the situation with Naphthol Yellow S (Tas et al 1978) As the molecular absorbance coefficient of Light Green is relatively high, objects with high protein content show local absorbances which are so high that they cannot be measured with any degree of accuracy Therefore, staining under non-optimal conditions is necessary in those cases

In view of the many similar properties of Light Green, Orange II, and Naphthol Yellow S a combination with the Feulgen method as described extensively for Naphthol Yellow S (Tas et al 1974, Gaub et al 1975) could be advocated for the other protein dyes as well As judged from its spectral characteristics (Fig 7 B), Light Green can be successfully combined with the Feulgen-Pararosanilin(SO_2) method In the case of Orange II, however, a combination with a Schiff type reagent with a spectrum lying more towards higher wavelengths seemed more desirable Therefore the combination with Feulgen-Thionin(SO_2) was studied Both in the case of the Feulgen-Pararosanilin(SO_2) Light Green and the Feulgen-Thionin(SO_2) Orange II combination the DNA staining proved to be unaffected by the simulated protein post staining procedure (Tables 3 and 4)

In both the DNA stainings either a sulfite water rinse or a running tap water rinse can be applied as the differentiation medium (Demalsy and Callebout 1967), without affecting the final result (results not shown) Although these differentiation media do not affect the Feulgen DNA values, in combination with the protein staining they strongly influence the latter In the case of Orange II a decreased protein staining is found that is most prominent when a sulfite water rinse was applied (Table 6) In the case of Light Green, descreased dye binding only is found

after using the sulfite water rinse (Table 5) The reason for these reductions is unknown, but it implies that the combined Feulgen-Thionin(SO_2) Orange II staining is unreliable for quantitative purposes For qualitative observations this combination may be of value, of course When the appropriate Feulgen-Pararosanilin(SO_2) Light Green procedure (with a running tap water rinse) is carried out in situ on different types of nuclei, the Feulgen-DNA measurements remain unaltered (Table 7) but, dependent on the type of nucleus, the Light Green values are altered though Both these findings are consistent with those found for the Feulgen-Pararosanilin(SO_2) Naphthol Yellow S staining (Table 8) In these two combination stainings a strong decrease (40–50%) is found in protein dye binding in nuclei with a rather condensed chromatin (chicken erythrocyte nuclei), whereas a slighter decrease (16% 20%) is observed in nuclei with a more dispersed chromatin (rat liver nuclei) The question may be raised whether this reduction could be related to a less quantitative staining of the proteins, thus reducing the validity of the combination stainings We think that this is not the case, at least for nuclei with comparable chromatin condensation For instance in rat liver nuclei a constant ratio in protein dye binding of 1 9 2 0 is found between tetraploid and diploid nuclei, both in individual stained as well in combined stained nuclei (see Tables 7 and 8) A greater steric hindrance especially in the more condensed chromatin by the Pararosanilin molecules bound to the DNA is a more likely cause for these reductions In any way it implies that protein dyes, that have proven to be quantitative reliable (see Figs 5B and C, and Tas et al 1974, 1978) will stain less protein in nuclei when they are combined with the Feulgen method

The reduction in Naphthol Yellow S staining of rat liver nuclei we found is somewhat higher than that found by Tas et al (1980) In the latter mentioned studies however no correction was made for the spectral overlap of Pararosanilin and Naphthol Yellow S at their respective peak wavelengths Indeed if the corrections in our data were omitted comparable results could be observed (results not shown)

In conclusion it can be stated that for the determination of DNA and protein in the same object the combined Feulgen-Pararosanilin(SO_2) Light Green can be applied, and is fully comparable to Feulgen-Pararosanilin(SO_2) Naphthol Yellow S As both Pararosanilin and Light Green match the sensitivity of the eye, cells stained according to this combined procedure facilitate the possibility of comparing a lightmicroscopically obtained pathological diagnosis with cytometric data of the same preparation

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SECTION 3.3

Flow cytometric analysis and sorting of human endometrial cells after immunocytochemical labeling for cytokeratin, using a monoclonal antibody

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Flow Cytometric Analysis and Sorting of Human Endometrial Cells After Immunocytochemical Labeling for Cytokeratin Using a Monoclonal Antibody[1]

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Endometrial cells in suspension were stained with propidium iodide and a monoclonal antibody against a cytokeratin intermediate filament protein specific for glandular and columnar cells (RGE 53). In this way columnar epithelial cells of the normal endometrium and of adenocarcinomas can be distinguished and separated by flow cytometry from non-epithelial cells (fibroblasts and inflammatory cells) and squamous epithelial cells, all of which are negative for RGE 53. This makes it possible to analyse and also sort pure fractions of this particular tissue type for further studies. The use of propidium iodide allows simultaneous DNA flow cytometry of these columnar epithelial cells. Therefore, the use of antibodies to cytokeratin in combination with propidium iodide can be of help in analyzing and sorting pure fractions of both normal and malignant cells. This allows a more refined examination of complex cell mixtures using flow cytometry.

Key terms: Cytokeratin, nucleic acid, endometrium, glandular cells, flow cytometry, flow sorting

Flow cytometry and image analysis are well-established procedures for quantitative cytophotometric determination of cellular constituents such as DNA, RNA, protein, and several specific antigens (9,10). However, benign and malignant tissues usually contain a significant number of stromal and inflammatory cells in addition to the tumor or other cell population of interest. Since this admixture of cells is highly variable both from tissue to tissue and within the same type of tissue, it is usually impossible to correct measurements, especially from flow cytometry (FCM), for this sample heterogeneity. As a result, measurements are often performed on complex mixtures of morphologically and proliferatively different cell types, which makes it difficult to interpret the data.

As one example, the endometrium of the uterus is composed of glandular ducts, lined by the columnar epithelial cells, and the intermingled stroma composed of fibroblasts and usually containing an assortment of blood cells. Adenocarcinomas of the endometrium originate from the columnar epithelial cells (4,18,21). Therefore, in order to study preneoplastic and neoplastic changes that produce this endometrial tumor type, it is obvious that measurements should be performed only on these epithelial cells.

Nucleated cells contain an intracellular matrix of fibrous proteins termed the cytoskeleton (12,14). A considerable part of the cytoskeleton is constituted by intermediate-sized filaments (IF), which have been proven to be tissue specific. These IF proteins are highly insoluble structures, often firmly bound to cell organelles (11). In general, cytokeratins are IF proteins specific for cells of epithelial origin and are absent from non-epithelial cell types. During carcinogenesis the IF protein type characteristic of the tissue of origin is retained, and normally no additional IF proteins are expressed (12,14). Polyclonal as well as monoclonal antibodies to IF proteins have, therefore, become valuable tools in tumor diagnosis and can be used in the recognition of different cell types within tumors (14).

We have recently described a monoclonal antibody (RGE 53) that specifically recognizes a cytokeratin polypeptide present only in glandular and columnar cells (15). In the present report we describe the use of this antibody, together with propidium iodide (PI) staining. Because a fluorescein isothiocyanate (FITC)-labeled second antibody is used for RGE 53 detection, the combi-

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nation with PI staining offers an easy method to select in a two-dimensional histogram the cells of interest In this way the DNA content of these cells can be determined or the cells can be sorted for further analysis

Recently a model system for this method has been described using mixed cell cultures of different origin (16) It could be shown that combined staining of cytokeratins and DNA permitted an accurate flow cytometric analysis of DNA content and distributions of the separate fractions within such combined cell populations Here we demonstrate that this method can also be applied to cell suspensions obtained from normal and malignant solid human tissue

MATERIALS AND METHODS

Preparation of the Cell Suspension

Normal endometrium obtained from fresh hysterectomy specimens (n = 28) by scraping the endometrial surface with a scalpel was immediately frozen in liquid nitrogen In case of neoplasms (n = 20), only tumor tissue was taken and frozen The remainder of the uterus was further processed for routine histology

For the preparation of the cell suspension, 0 2–0 4 g of tissue was quickly brought to 4°C and suspended in phosphate buffered saline (PBS, 140 mM NaCl, 8 mM Na_2HPO_4 $2H_2O$, 1 67 mM NaH_2PO_4 H_2O, pH 7 2) containing 1 mM EDTA After gentle homogenization (three strokes with a loose fitting pestle in a Dounce homogenizer), cells were centrifuged for 10 min at 250g, and the resulting pellet resuspended in 5 ml PBS containing 5 mM EDTA Cell clusters were further disrupted by incubating the suspension at 37°C for 1 h with occasional syringing (three times four strokes through a 1 1 mm needle during the first half hour and three times three strokes through a 0 8 mm needle during the second half hour) Thereafter the cell suspension was injected into 15 ml of cold (−20°C) methanol, centrifuged at 4°C for 10 min at 250 g, and the pellet resuspended in 2 ml of cold (−20°C) methanol Cell concentration was determined using a Coulter Counter Model ZB1 (Coulter Electronics, Hialeah, FL) and chicken erythrocytes (fixed in methanol) were added in a 1 10 ratio, as an internal standard (6,19) The suspension was kept at 4°C until staining

Immunocytochemical Staining Procedure

Preparation and specificity testing of the monoclonal antibody to cytokeratin (RGE 53) has been described previously (15) The immunocytochemical staining procedure was performed at room temperature as follows The cell suspension, obtained as described above, was centrifuged for 10 min at 250g, and the pellet washed twice with PBS containing 1 mM EDTA and 5% foetal calf serum (Gibco, Paisley, UK) by 10 min centrifugation steps at 450g Thereafter the cell pellet was incubated in 400 μl of RGE 53 culturing supernatant for 30 min At the end of the incubation period 4 ml of the aforementioned PBS solution was added, and the suspension was centrifuged for 7 5 min at 450g and washed once There after the cells were incubated in 400 μl of an FITC conjugated goat-anti mouse or rabbit anti mouse IgG antibody solution (diluted 1 12 5, Nordic, Tilburg, The Netherlands) for 30 min After two subsequent washing steps the cell pellet was resuspended in 1 ml PI (A grade, lot No 910280, Calbiochem, La Jolla, CA) solution (2 μg/ml in 75 mM NaH_2PO_4 H_2O, 75 mM Na_2HPO_4 $2H_2O$, pH 7 0) It was incubated for 10 min and filtered through a 50 μm filter (Van Wijk Industrie Producten, Santpoort, The Netherlands) For DNA flow cytometry, RNase digestion was performed before PI staining by incubating the cells 10 min at 37°C in 0 1% (w/v) RNase (Type I AS, Sigma, St Louis, MO) in PBS containing 1 mM EDTA For cell sorting the RNase treatment was omitted

For control experiments the cells were incubated with RKSE 60, a monoclonal antibody specific for keratin intermediate filament protein (IFP) of keratinizing stratified epithelia and keratinizing cells in squamous cell carcinomas Preparation and specificity testing of this monoclonal antibody has been described elsewhere (14,17) In brief the antibody does not react with columnar or glandular epithelia, nor with non-epithelial tissue All labeling conditions were the same as with RGE 53 staining For both monoclonal antibodies, culturing supernatants, known to give strong reactions in the indirect immunofluorescence technique, were used for the labeling of the cell suspensions

Flow Sorting

Cell analysis and sorting was performed using a Cytofluorograph system 50 H (Ortho Instruments, Westwood, MA) Fluorochromes PI and FITC were excited by light of 488 nm wavelength from an argon ion laser (Spectra Physics, Mountain View, CA) Fluorescence was measured simultaneously using a 515–530 nm filter and a longpass 630 nm filter for FITC and PI, respectively A window was placed interactively in the two-dimensional histogram (nucleic acid as PI versus cytokeratin as FITC) A total of 5,000–10,000 of the cells of interest (FITC and PI positive) were sorted into 21 μl Carbowax (2% polyethylene glycol 1500 solution in 50% ethanol) or into 21 μl of a 0 25% formaldehyde solution on xylene cleaned glass slides After air drying, the cells collected in Carbowax were fixed in 100% methanol, and after a descending series of methanol, they were rinsed in distilled water and mounted in 50% glycerol in PBS The cells collected in formaldehyde were stained with Feulgen Pararosanilin(SO_2) Light Green (13)

RESULTS

The specificity of the reaction of the monoclonal antibody RGE 53 with human endometrium is shown in Figure 1 Figure 1a shows normal endometrium in which the glandular and columnar cells show a bright fluorescence staining, while the surrounding connective tissue fibroblasts and blood cells are negative Figure 1b shows a moderately differentiated adenocarcinoma Note that the number of positive cells has increased considerably

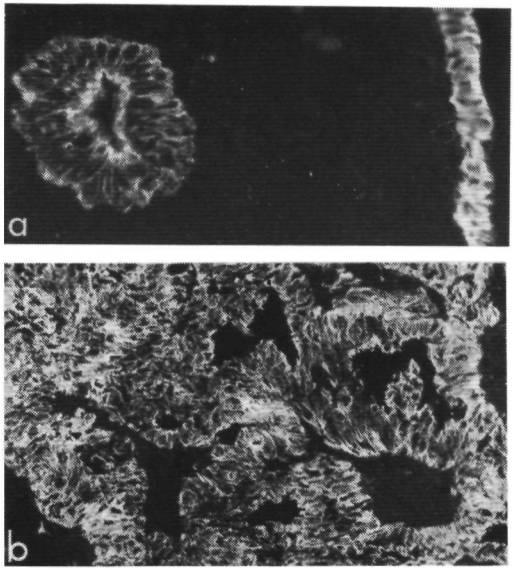

FIG. 1. a. Immunofluorescence photomicrograph of normal endometrium stained with RGE 53, a monoclonal antibody specific for cytokeratin of glandular and columnar epithelial cells (×200). b. Immunofluorescence photomicrograph of a moderately differentiated adenocarcinoma of the endometrium, stained with RGE 53 (×160).

They are aligned in irregularly shaped, closely packed glands.

When cells isolated from the endometrium and stained for cytokeratin and DNA were analyzed by flow cytometry, several cell populations could be distinguished. Figure 2A shows a three-dimensional histogram obtained by analysis of cells from normal endometrium. Cells positive for cytokeratin can be distinguished clearly from negative cells. Furthermore, PI staining distinguishes cells containing nuclei from cell debris and loose cytoplasmic fragments. Finally a DNA histogram of the cytokeratin positive cells can readily be obtained from these data. In this material the position of the G0/G1 peak was 2.7 times higher than that of chicken erythrocytes with a coefficient of variation less than 6.0%.

The influence of RNase treatment on PI staining was studied in several normal and abnormal endometrial specimens. Omitting RNase resulted in less than 6% change in the position of the G0/G1 peak and an increase in the coefficient of variation of this peak of less than two percentage points. The presence or absence of the RNase treatment also had no significant influence on the RGE 53 staining. Because RNase treatment had a significant effect on other measurements on sorted cells (manuscript in preparation) this was omitted when cells had to be sorted.

For cell sorting a window was placed interactively (Fig. 2B) allowing analysis and sorting only of FITC- and PI-positive cells. In the last category only cells falling within the G0/G1 to G_2M range were sorted because cells of other ploidy are not found in normal endometrium (2,18). A mean of 20.7% (SD = 9.0%) positive cells was found in this way for 28 normal endometria.

Similar studies were performed with neoplastic lesions of the endometrium. Figure 2D shows a three-dimensional histogram of cells from a well differentiated adenocarcinoma of the endometrium. Again it can be seen that epithelial cells (RGE 53 positive) can be clearly distinguished. In the sample illustrated, the main fraction of the cells is found in the diploid region. When a window is placed interactively in this histogram (see Fig. 2E), RGE 53-positive nucleated cells can be separated from RGE 53-negative objects and cell debris. A mean of 33.5% (SD = 17.0%) positive cells was found for 20 adenocarcinomas.

The correctness of the window was checked, before the actual sorting procedure started, by sorting a small sample (1,000 cells) onto a slide. This was examined for FITC and PI fluorescence by fluorescence microscopy.

RGE 53 is an IgG1 with a kappa light chain (14,15). As a control for the specificity of the labeling another monoclonal antibody of the same IgG class, RKSE 60, was studied. RKSE 60 specifically recognizes keratin IFP in keratinizing stratified epithelia and keratinizing cells in squamous cell carcinomas (14,17). As can be seen in Figures 2C and F, both normal endometrium and the endometrial adenocarcinoma are negative for this antibody. Figure 3a shows an immunofluorescence picture of an unselected, mixed population of cells obtained after staining of a normal endometrium preparation. In this preparation both RGE 53-positive and RGE 53-negative cells are found in addition to naked nuclei and cell debris. After sorting of the cells, only RGE 53 and PI-positive cells were found when the preparation was examined by fluorescence microscopy (Fig. 3b). Figure 3c shows an unselected mixed population of cells of a well differentiated adenocarcinoma and Figure 3d the cells after sorting. Cell morphology after sorting was well preserved as observed after Feulgen-Pararosanilin(SO_2) Light Green (13) or routine cytological staining.

DISCUSSION

The present study demonstrates the use of cytokeratin antibodies in indirect immunofluorescence to identify glandular and columnar epithelial cells in mixed cell populations from normal human endometrium and human endometrial adenocarcinoma using flow cytometry. In this way inflammatory cells, fibroblasts, and squamous epithelial cells can be excluded from analysis. No significant differences in RGE 53 staining insensity has been found between normal and carcinoma specimens, indicating that normal columnar cells cannot be discrimated from their malignant counterpart based on RGE 53 staining alone. Since FITC is used for the immunofluorescence channel, simultaneous FCM analysis of cellular nucleic acid content can be performed if the cell

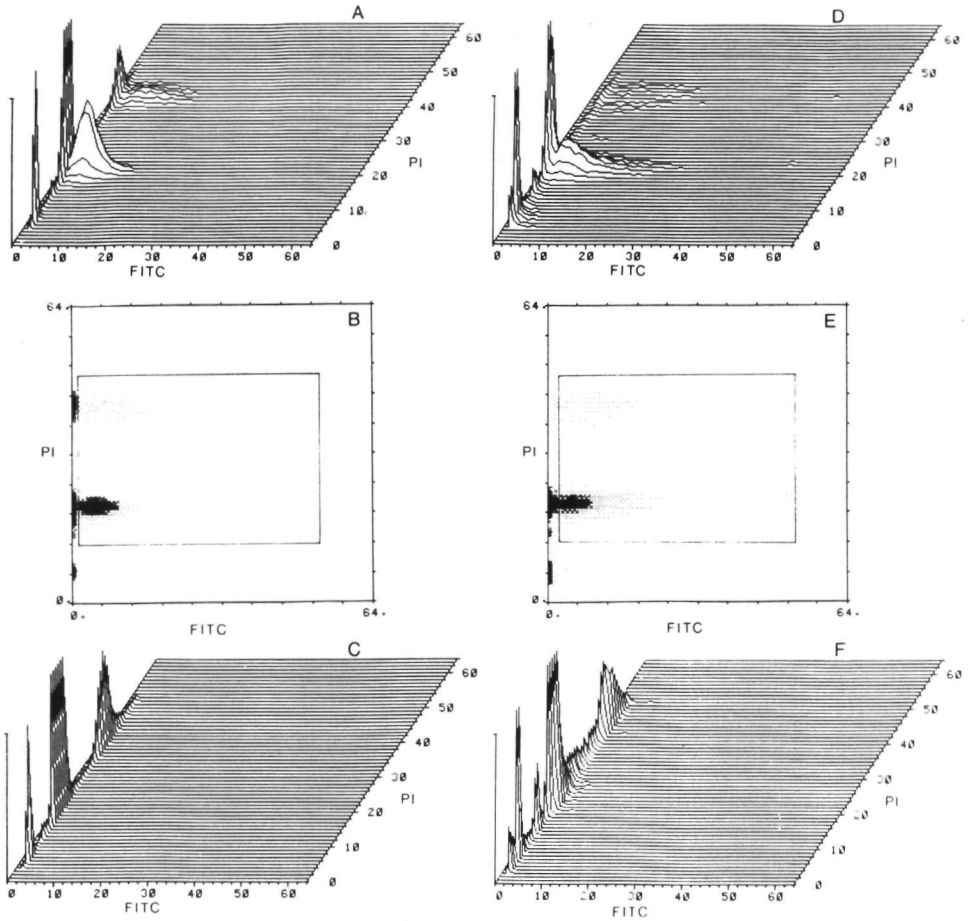

FIG. 2. FCM analysis of cells from normal endometrium (A,B,C) and a well-differentiated endometrial adenocarcinoma (D,E,F) stained with PI for DNA (ordinate) and FITC indirect immunofluorescence of glandular and columnar cell cytokeratin (abscissa in A,B,D, and E) or stratified squamous epithelial cell cytokeratin (abscissa in C and F). Limits of PI- and FITC-positive populations are indicated by sorting windows in B and E. On the ordinates, the first peak represents the chicken erythrocyte singlet internal standard and a second smaller one (especially well visible in F) chicken erythrocyte doublets. In A,B,D, and E, the G0/G1, S, and G2M regions contain cells both positive and negative for columnar cell cytokeratin. In C and F only cells negative for stratified squamous epithelial cell cytokeratin are found.

suspension is also stained with propidium iodide (3,5). This technique is analogous to that developed by Braylan and associates (1) for analyzing human lymphoid cell populations. These DNA-histograms thus allow the detection of a heterogeneity of the columnar cell population in carcinoma specimens. Most carcinoma specimens show a G1/G0 peak in the diploid region which is shifted to slightly higher values compared to the chicken erythrocyte standard (ratio 2.8–3.0; manuscript in preparation). These peaks are unimodal, and no significant change in the coefficient of variation is noted as compared to normal specimens. This indicates that the malignant columnar cell population is rather pure, although minor contamination with normal cells cannot be completely excluded.

Because of the ubiquitous presence of cell-type-specific intermediate filaments, immunofluorescence of these structures can be used for FCM analysis of many solid tumors. Carcinoma cells can be distinguished from non-epithelial cells by broad-spectrum cytokeratin antibodies (recognizing all types of epithelial cells), and adenocarcinoma cells from non-glandular epithelium and non-

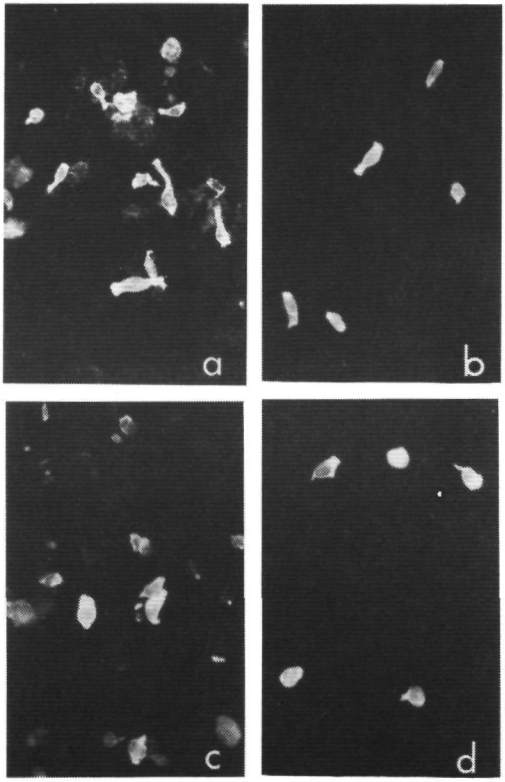

FIG. 3. Immunofluorescence photomicrograph of single endometrial cells stained for nucleic acid (PI) and glandular cytokeratin (FITC). a. Unsorted population of normal endometrium. b. PI- and FITC-positive cell population sorted according to the window depicted in Figure 2B. c. Unsorted population of cells of a well-differentiated adenocarcinoma. d. PI and FITC-positive cell population sorted according to the window depicted in Figure 2E. a–d: ×200.

epithelial elements by more specific (monoclonal) antibodies like RGE 53 (14,15). In addition, antibodies specific for muscle cells (anti-desmin), neural cells (anti-neurofilament), and glial cells (anti-glial fibrillary acidic protein, GFAP) and the respective tumors derived from these cell types are available (12,14). These tissue markers allow specific recognition, analysis and sorting of specific cell types, excluding stromal and inflammatory elements.

Together with PI-RGE 53-positive and -negative cells, a variable number of naked nuclei were present in the samples used for the present study. Thus the present technique may exclude from analysis some columnar and glandular epithelial cells which have lost their cytoplasm. Thus loss of some epithelial cells from analysis is possible, as is true of FCM analysis in general (7,8). However, use of the present method does ensure that FCM analysis of nucleic acid content and sorting is performed only on glandular and columnar epithelial cells.

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CHAPTER IV

MEASUREMENTS IN HUMAN ENDOMETRIUM

SECTION 4.1

DNA and nuclear protein measurements in human endometrium

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Feulgen-DNA and nuclear Light Green-protein measurements have been performed in isolated nuclei of normal (non malignant) and malignant human endometrium.

The DNA content of the GO/G1 fraction of malignant endometrium showed much overlap with that of normal endometrium, or was slightly increased. Two of the eighteen carcinomas were clearly aneuploid. No correlation was found between the histological grade and the DNA content. The tumors of clinical stage II and higher all had a higher DNA content than normal endometrium.

The percentage of cells present in the proliferative fraction was higher in proliferative endometrium than in secretory and postmenopausal atrophic endometrium. For malignant endometrium percentages were found comparable to that of normal endometrium or higher. No correlation was found with the histological grade. Tumors of stage II and higher had intermediate values compared to carcinomas below stage II.

The nuclear protein/DNA ratio of malignant endometrium completely overlapped that of normal endometrium. However, when the age of the patient was taken into consideration, most values of the carcinomas exceeded that of normal endometrium. Within the tumor population no correlation was found with the histological grade. Higher values were found with tumors of clinical stage II and higher.

Since neoplastic development is reflected by changes in the cell nucleus, analytical cytologic measurements have particularly been focused on the nucleus. Morphologic parameters such as nuclear size (14) and texture (14,36) have been determined, but most emphasis has been put on quantitative DNA-measurements (3,7,9,11,18,20,22,27). In many cases tumor development is accompanied by evident deviations in this DNA-content and therefore the relationship has been sought between these two entities to elucidate prognostic parameters (3,7,20,22,30).

Besides DNA, nuclear protein constitutes the major macromolecular mass of the nucleus. During cell development the amount of this protein varies. For instance biochemical (21,25,37) and cytochemical (4,5,33,35,45) data have shown that at the onset of DNA synthesis a considerable increase in nuclear nonhistone protein (NNHP) is noted. An increased NNHP/DNA ratio is therefore usually found in proliferating tissue (28,30). Also for malignant tissues both biochemical 1,10,13,28,29,38,39) and cytochemical (6,12) studies have reported an increased NNHP content compared to normal tissue. As a possible explanation for this increase the new proliferative state of the cells is mentioned (6,39). Auer and coworkers (6) therefore suggest that the amount of nuclear protein could be used for malignancy grading.

So far nuclear cytometric measurements of malignant changes in the endometrium have been focussed upon the determination of the DNA content. These measurements have been performed on glandular cells in tissue sections (24,30,44) on isolated cells (11,42) and isolated nuclei (17). The emphasis in these studies has been placed on ploidy measurement (11,24,30,42,44), that is the DNA-content of the GO/G1 fraction, and the determination of cells in the proliferative fraction (17,42).

In the present study scanning cytometric DNA and nuclear protein measurements have been performed on isolated nuclei from normal and malignant endometrium. Emphasis has been placed on the determination of the DNA content of the GO/G1 fraction, the percentage of cells in the proliferative (DNA synthesizing) fraction and the nuclear protein content.

Clinical material

Normal endometrium and endometrial tumor material were obtained from fresh hysterectomy specimens. Normal endometrium cells were scraped from the underlying myometrium and immediately frozen in liquid nitrogen. The remainder of the uterus was processed for routine pathology. In case of a tumor, material was taken directly from the tumor and cut in small (about 2 x 2 x 2 mm) pieces. One piece was processed together with the rest of the uterus for routine pathology. This was done to compare the histology of the tissue used for cytometry with that of the remainder of the tumor in the uterus. The rest of the tumor material was frozen and stored in liquid nitrogen for later nuclear preparation.

For normal endometria the period of the menstrual cycle was obtained from clinical information and confirmed by routine pathology. Normal endometrium was obtained from patients with cervical intraepithelial neoplasis (3), menorrhagia(7), myoma (7), prolapsis (3) or other geneto-urinary problems (4). The endometrial tumors were diagnosed by two pathologists. Histological grading was according to the World Health Organization classification (34) in grade 1 (well-differentiated), grade 2 (moderately differentiated) and grade 3 (poorly differentiated). Care was taken that the grade of the small piece of tumor taken for cytometry corresponded with that of the tumor present in the uterus. If this part of the tissue in case of mixed grading of the tumor, contained only one of the components, the grade of this piece was taken. Clinical staging was according to the criteria of the International Federation of Obstetrics and Gynecology in stage I to IV. In table I a summary of the tumor material is shown.

Nuclear isolation

About 0.2 g of the liquid nitrogen stored material was quickly brought to $4^{o}C$ and suspended in 5 ml 140 mM NaCl, 3mM KH_2PO_4, 13mM $Na_2HPO_4.2H_2O$, 1mM EDTA, 1mM phenylmethylsulfonylfluoride pH=7.2. The nuclear suspension was prepared by homogenization using

Table I. Patient material with endometrial adenocarcinoma.

No	Age	Histological grade	Clinical stage
1	75	1	IA
2	55	1	IB
3	50	1	IA
4	64	1	IA
5	69	1-2	III
6	78	1-2	IA
7	44	1-2	IB
8	72	1-2	IA
9	63	1-2	IB
10	86	2	IB
11	73	2	IA
12	59	2	III
13	60	2	IA
14	71	2	IB
15	69	2-3	IB
16	74	a	II
17	57	a	III
18	61	a	IV

a = adenosquamous carcinoma

a Dounce homogenizer with three strokes of a tight fitting pestle. This suspension was further processed by one of the following two procedures:

a) The nuclear suspension was poured into a funnel, with a polycarbonate membrane (Nuclepore, Pleasanton, USA) in the bottom containing 5 μm diameter pores. This suspension was washed twice at 4°C with equal volumes of the above mentioned solution, and drained through the membrane under unit gravity. The washed suspension was collected and the number of nuclei determined using a Coulter Counter Model ZB1 (Coulter Electronics, Hialeah, USA). After centrifugation (10 min x 500 g) the nuclear pellet was resuspended in 2% polyethylene glycol 1500 in 50% ethanol. Thirtyfive microliter containing approximately 40.000 nuclei, was brought onto a glass slide, air dried and fixed in a methanol, 37% formaldehyde, acetic acid mixture (85 : 10 : 5, v/v/v) for one hour.

b) The nuclear suspension was injected directly into the same fixation mixture (ratio nuclear suspension/fixative = 1 : 15). After one hour of fixation the number of nuclei was determined and approximately 40.000 nuclei were poured into a funnel with a polycarbonate membrane tape in the bottom in a device as described elsewhere (32). Nuclei were collected on the mebrane by filtration and then automatically transferred to a glass slide by pressure-fixation. Post-fixation of a half hour in the aforementioned fixation mixture was routinely applied.

The latter preparation procedure was found to be an improvement of the first one in terms of speed and ease of handling. No significant differences in the measuring results were obtained after using either of both procedures. After a descending series of methanol, the slides were rinsed in deionised water, followed by 0.1M phosphate buffer pH=7.0, and deionised water again and then air dried. They were stored at 4°C until staining.

Staining and measurement

The nuclei were stained for DNA and protein using Feulgen-Pararosanilin(SO_2) Light Green (31) and mounted in a

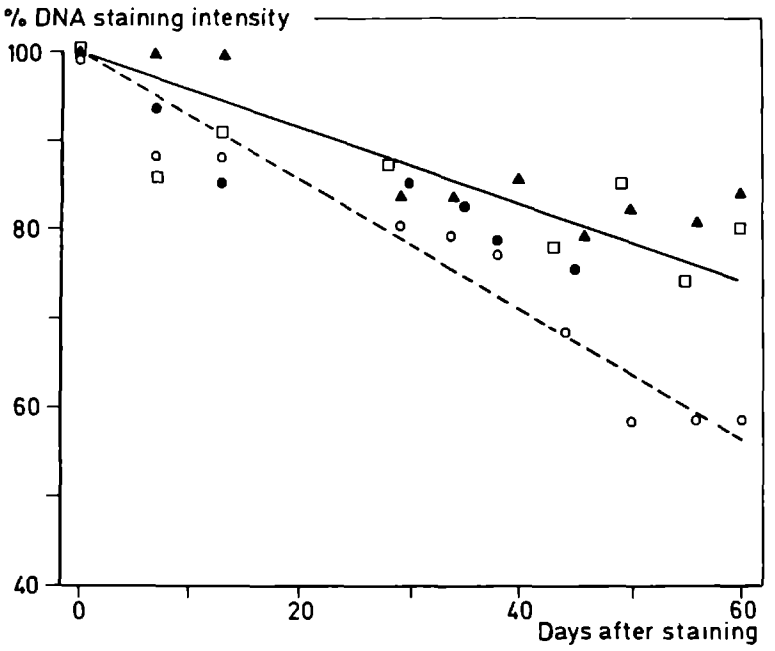

Figure 1. Time dependency of the Feulgen-DNA staining intensity. The DNA content of the G0/G1 fraction of a normal endometrium (▲), an adenosquamous carcinoma (●) a moderately differentiated adenocarcinoma (□) and chicken erythrocytes (○) were measured on different days after staining, and the numbers related to that measured on the day of staining. A line was fitted for the endometrial material (—) and the chicken erythrocytes (- -). Different slopes were found for the decrease in DNA staining intensity of the more dispersed chromatin in the endometrial nuclei and the compact chromatin in the chicken erythrocyte nuclei.

synthetic resin (Permount, Fisher Scientific Co, USA). A slide with chicken erythrocyte nuclei was stained together with the endometrium nuclei as a control for possible staining variations. Not later than two months after staining 100 nuclei equally distributed over two slides were measured at random using an Axiomat scanning microscope (Zeiss, Oberkochen, W-Germany). Nuclei that still had clearly visible cytoplasmic fragments and also nuclei of inflammatory cells

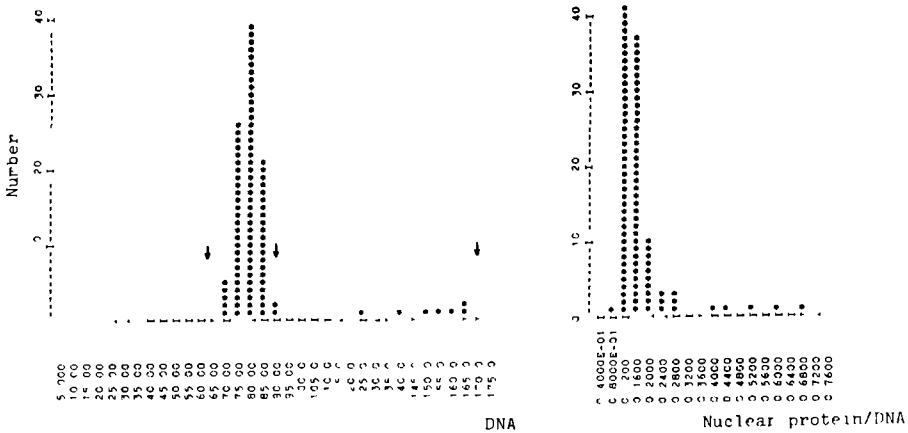

Figure 2. Feulgen-DNA (left) and nuclear Light Green protein/Feulgen-DNA (right) histogram of a moderately differentiated adenocarcinoma, stage IA. In the DNA histogram the position of the GO/G1 peak is interactively found and indicated between the arrows at the left and the proliferative (S and G_2M) fraction at the right. Because it is difficult to indicate the exact position of the border between the GO/G1 and the S-phase the minimal and maximal percentage of cells in the proliferative fraction are given: 7-9%.

were not measured. The latter nuclei did not have a discernable chromatin texture, and were smaller than the endometrial cell nuclei. No attempts were made to discriminate tumor cell nuclei from non-tumor cell nuclei, in preparations obtained from adenocarcinomas. There were no reliable morphological criteria that would have supported this discrimination. However in the histological sections made from the tumor tissue used for cytometry, at least 50% tumor cells were found.

Measurement of DNA and nuclear protein was performed as described elsewhere (31). Because the DNA-staining intensity decreased during storage (Figure 1), the measurements were corrected according to the slope found in Figure 1 and calculated for the day of staining. A Feulgen-DNA histogram and a nuclear Light Green-protein/Feulgen-DNA histogram were plotted on the basis of these corrected measurements. A typical example is shown in Figure 2. In the DNA histogram the position of the Go/G1 peak and the percentage of cells in the

proliferative fraction (S and G_2M) were then determined interactively. In the nuclear protein/DNA histogram the mean was calculated.

RESULTS

In Figure 3 histograms are shown of the DNA content of the GO/G1 fraction of normal and malignant endometrium. As can be seen an overlap is noted between both populations, with that of the malignant showing a tendency towards higher values. Eight cases are slightly higher while two cases show a considerable increase in DNA-content compared to normal endometrium. On basis of this DNA-content these last two can be categorized as triploid (adenocarcinoma grade 1-2, stage III) and pentaploid (adenosquamous carcinoma, stage II). In the histogram of the normal endometrium the values for the different phases in the menstrual cycle are found scattered. In the histogram of the malignant endometrium no clear correlation is found between the histological grade and the DNA content, except for the adenosquamous carcinomas, which tend towards higher values. When only the clinical stage is taken into account it can be seen in Figure 3C that the cases with stage II and higher all have a higher DNA content than normal endometrium in Figure 3A. ($P < 0.002$, Wilcoxon 2-sample test) and than most lower staged tumors ($P < 0.005$, Wilcoxon 2-sample test).

The percentage of cells present in the proliferative fraction are shown in Figure 4. In this figure the minimal and maximal numbers are indicated per case because it is impossible to indicate exactly where the GO/G1 fraction ends and the S fraction starts. As could be expected in the proliferative endometrium a higher number of cells (6-17%) is present in the proliferative fraction than in secretory (0-8%) or postmenopausal atrophic endometrium (0-7%). In malignant endometrium these numbers vary considerably. Cases with virtually no

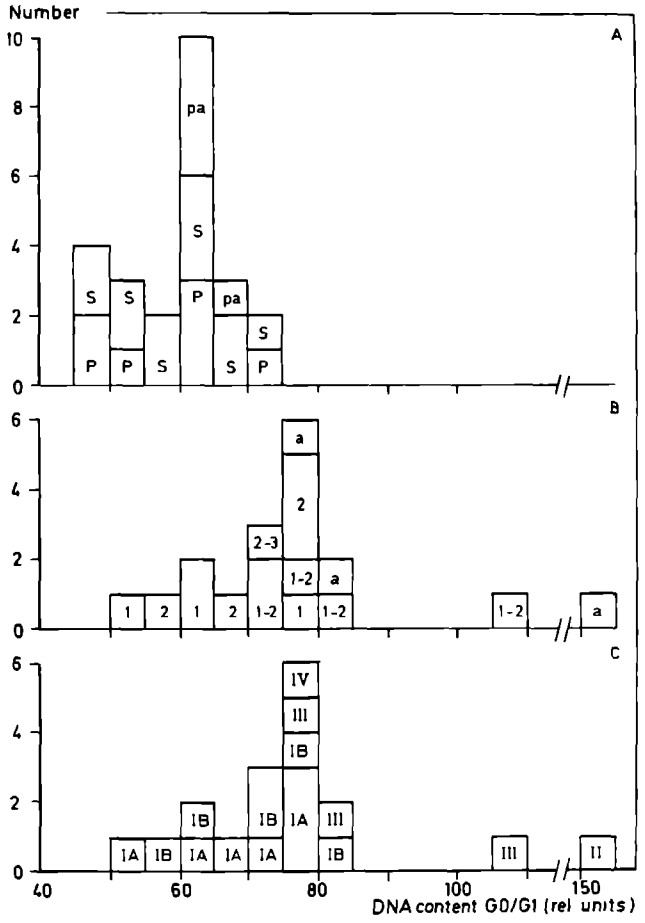

Figure 3. Histograms of the Feulgen-DNA-content of the GO/G1 fraction of normal (A) and malignant endometrium, classified according to histological grade (B) and clinical stage (C). In A "p" indicates proliferative, "s" secretory and "pa" postmenopausal atrophic endometrium. In B the grade and in C the stage are indicated in the bars; in B "a" indicates adenosquamous carcinoma.

cells in the proliferative fraction were found, but also cases with very high numbers, up to 23%. No clear correlation is found between the histological grade and the percentage of cells in the proliferative fraction. When compared to the clinical stage we see

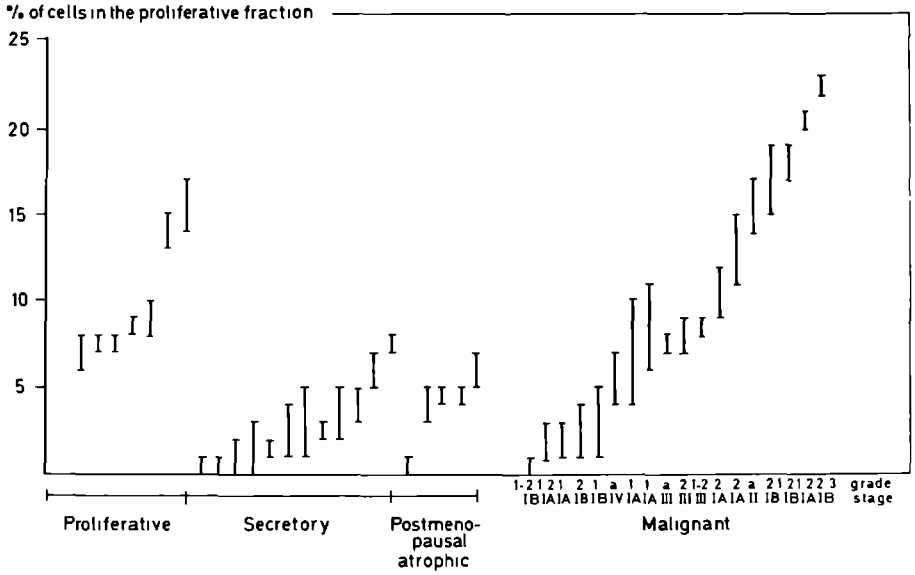

Figure 4. Percentage of cells in the proliferative fraction of proliferative, secretory, postmenopausal atrophic and malignant endometrium. The minimal and maximal number per case are indicated. For malignant endometrium the number for grade and stage are indicated on the abscissa; "a" indicates adenosquamous carcinoma.

that the cases with stage II and higher are found in the "intermediate" zone.

Figure 5 shows histograms for the nuclear protein content which is normalized for DNA of normal and malignant endometrium. As can be seen, there is a complete overlap between both histograms. In the histogram of normal endometrium the values for postmenopausal atrophic endometrium are lower than those found for proliferative and secretory endometrium. No difference can be found between the latter two. In the histogram of malignant endometrium the values for the different histological grades are scattered (Figure 5B). When clinical stage is considered it can be seen that four of the five of the clinical stages II and higher are found towards higher values (Figure 5C).

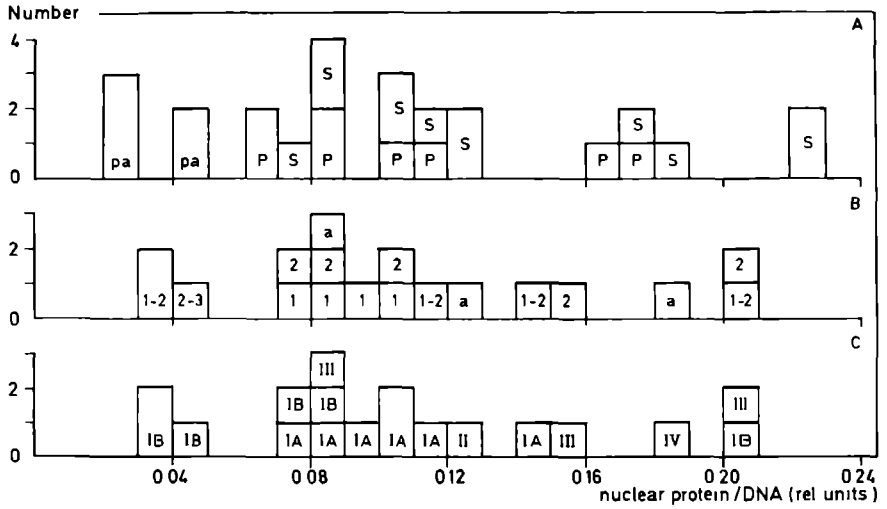

Figure 5. Nuclear Light Green protein/Feulgen-DNA histograms of normal (A) and malignant endometrium, classified according to histological grade (B) and clinical stage (C). For further details see Figure 3.

DISCUSSION

In the present study DNA and nuclear protein measurements performed on normal (non-malignant) and malignant endometrium were compared. The distribution of the DNA content of the GO/G1 fraction of malignant endometrium compared to that of normal endometrium agrees with the data found by others. Atkin (2), Hustin (24) and Feichter et al (17) mention that most adenocarcinomas in their studies fall within the normal diploid region. Böhm and Sandritter (11) report a slightly increased DNA content for most carcinomas, 2.5C compared to the 2C standard for human lymphocytes. Probably this value is somewhat overestimated as a consequence of underestimation of their standard. This means that the value for these tumors must be found more close to that of normal endometrium, which again corresponds with our results. Wagner et al (44) mention aneuploid DNA distributions for the tumors in their study. These data should

however be interpreted with some caution because the measurements were performed on tissue sections using socalled "plug" measurements. These data are far less accurate than scanning cytophotometric measurements on isolated intact nuclei, as performed here. The found data are also in agreement with cytogenetic data (8) which show chromosome counts ranging from 46 to 49, which would correspond with a normal to slightly increased DNA content.

Hustin (24) and Feichter et al (17) report that all of their well-differentiated (grade 1) tumors have diploid DNA values. Also in this study the grade 1 tumors fall within the diploid region, but also a number of the tumors diagnosed as less differentiated. Two of the tumors in this study were clearly aneuploid. Feichter et al (17) report that three of their eleven tumors showed marked aneuploidy. From the data of Böhm and Sandritter (11) it can be concluded that three of the eight tumors in their study were clearly aneuploid.

In this study the tumors of stage II and higher had a higher DNA content than normal endometrium, and most lower staged tumors. Although the number of tumors is relatively small (13 stage I against 5 of stage II and higher) this tendency agrees with that found with other tumor types; namely an increase in DNA content (or aneuploidy) with increasing stage of the tumor (9,27).

The percentage of cells in the proliferative fraction in the higher staged adenocarcinomas was comparable to that of normal proliferative endometrium and was not significantly increased compared to that of the other tumors. The numbers found were comparable to those reported with flow cytometric techniques where values between 5 and 11% have been found for well differentiated carcinomas (17).

The staining procedure used in this study allowed next to DNA measurements, also the determination of nuclear protein. Biochemical (1,10,13,28,29,38,39) and cytochemical (6,12) studies gave rise to the expectation that this parameter could be useful in the detection and grading of malignancies. However results of these measurements should be interpreted with more caution, than those of DNA. The latter is firmly bound in the nucleus and not easily removed by isolation (23,40,41), fixation (19,43) and staining procedures (15,16,43). Nuclear protein, however, is more losely bound and therefore more sensitive to loss in aforementioned procedures (19,23). Although the conditions were chosen to minimize this

effect, the results in this study may be interfered by a certain nuclear protein loss.

The nuclear protein content in malignant endometrium is not significantly elevated compared to normal endometrium, when proliferative and secretory endometrium are included. Most (16 of the 18) adenocarcinomas are found however amongst elderly postmenopausal women (see Table I). The nuclear protein content in normal atrophic endometrium for this group is low and most adenocarcinomas exceeded these values. The tumors that showed pronounced invasive growth or metastases (grade II and higher) are found in the high nuclear protein region. These data seem to support the observations of Auer and coworkers (6,12), from studies on intact breast carcinoma cells, that nuclear protein could be used as a measure for maligancy grading.

In conclusion it can be stated that most adenocarcinomas have the same or a slightly increased DNA content, compared to normal endometrium. The number of tumor cells present in the proliferative fraction varies considerably and is not related to histological grading or clinical staging. Nuclear protein may be a useful parameter in the further grading of malignancies. However, in order to determine such a relationship in more detail, more tumor material with longer follow-up will be necessary.

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SECTION 4.2

DNA and nuclear protein measurements in columnar epithelial cells of human endometrium

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Propidium iodide DNA flow cytometry, Feulgen-DNA and nuclear Light Green protein scanning cytometry have been performed in columnar epithelial cells of normal, non malignant, human endometrium and endometrial adenocarcinomas.

DNA measurements derived from flow and scanning cytometry showed comparable results. The DNA content of the GO/G1 fraction of the adenocarcinomas had a considerable overlap with that of normal endometrium, with that of the carcinomas shifted towards higher values. For the carcinomas no correlation was found with the histological grade, with the exception of the adenosquamous carcinomas. Most of the clinical stage I tumors showed a DNA content in the normal diploid region. Three of the four carcinomas of clinical stage II and higher had an increased DNA content.

For the carcinomas the percentage of cells in the proliferative fraction, as determined from scanning cytometric derived DNA histograms, were comparable to that of normal endometrium, or higher. No correlation was found with the histological grade. Tumors of clinical stage II and higher had intermediate values compared to carcinomas of lower stages.

The nuclear protein/DNA ratio of malignant endometrium completely overlapped that of normal endometrium. Within the tumor population no correlation was found with the histological grade, with exception of the adenosquamous carcinomas, and clinical stage.

Based on aforementioned parameters no discrimination could be obtained between normal and malignant endometrium. However when the DNA content of the GO/G1 fraction was combined with the coefficient of variation of the nuclear protein/DNA ratio a clear discrimination could be obtained with only two false positive cases.

In a preceding paper (11) we have compared DNA and nuclear protein values in normal endometrium and endometrial adenocarcinomas to study the changes herein during neoplastic transformation. The results showed that the carcinoma cells had a the same or an increased DNA content compared with normal endometrium, a large variation in the percentage of cells in the proliferative fraction and a comparable amount of nuclear protein. These measurements were performed in isolated nuclei obtained from homogenates of normal endometrium and endometrial adenocarcinomas. Normal endometrium and endometrial adenocarcinomas are composed of several tissue types, however. Normal endometrium contains of a varying number of glands, dependent on the period of the menstrual cycle, which are lined up by columnar epithelial cells. The glands are surrounded by stroma and blood vessels. Endometrial adenocarcinomas are derived from this columnar epithelial cell type and are characterized by a considerable increase in the number of glands (6,14,15). Thus former measurements were performed in a mixture of nuclei, derived from epithelial as well as mesenchymal cell types. A comparison between measurements in the nuclei of the columnar epithelial cells of normal endometrium and adenocarcinomas should therefore give more appropriate information.
Recently we have developed a staining procedure, enabling the specific recognition of columnar epithelial cells in cell suspensions from normal and malignant endometrium (10). This technique is a combination of the indirect immunofluorescence detection of cytokeratin 18, an intermediate filament protein specifically present in glandular and columnar cell epithelium (12), together with propidium iodide (PI) staining of DNA. The DNA-content of these cells can then be measured flow cytometrically and the cells can also be sorted on glass slides for later scanning cytometry.
In this paper results are presented from DNA flow cytometry and DNA and nuclear protein scanning cytometry performed in columnar epithelial cells from normal endometrium and endometrial adenocarcinomas.

Normal endometrium and endometrial adenocarcinomatous tissue were obtained from fresh hysterectomy specimens. Normal endometrial tissue was scraped with a scalpel from the underlying myometrium. From adenocarcinomas fragments were cut in small (2 x 2 x 2 mm) pieces. Scraped normal tissue and the small tumor fragments were immediately frozen in liquid nitrogen. The remainder of the uterus and, in case of a carcinoma, also one small tumor fragment was processed for routine histopathologic diagnosis.

Normal endometrium was obtained from patients with cervical intraepithelial neoplasias (3), menorrhagia (4) fibroids (9), uterine prolaps (2) or other (7) genito-urinary problems. These patients, as well as those with adenocarcinomas, had not received any hormonal medication including oral contraceptives. The phase of the menstrual cycle was established by anamnesis and confirmed by routine histopathologic diagnosis. The endometrial carcinomas were diagnosed by two pathologists. Histological grading was performed according to the World Health Organization classification into grade 1 (well-differentiated), grade 2 (moderately differentiated) and grade 3 (poorly differentiated). Care was taken that the histological grade of the piece taken for cytometry corresponded with that of the tumor present in the uterus. If this part of tissue, in case of mixed grading, contained only one of the components, the grade of this piece was taken.

Clinical staging was according to the criteria set by the International Federation of Gynecology and Obstetrics into stage I to IV.

In table I a summary is shown of the tumor material.

Single cell preparation and the staining of the cells for DNA with PI and for cytokeratin 18 with the monoclonal antibody RGE 53 have extensively been described elsewhere (10). Briefly single endometrial cells were obtained by gentle homogenization, followed by syringing in a PBS solution containing 5 mM EDTA. These cells were fixed in cold methanol and stained for cytokeratin 18 using the indirect immunofluorescence technique with a fluorescein isothiocyanate (FITC) labeled rabbit-anti-mouse IgG second antibody.

Table I. Patient material with endometrial adenocarcinoma.

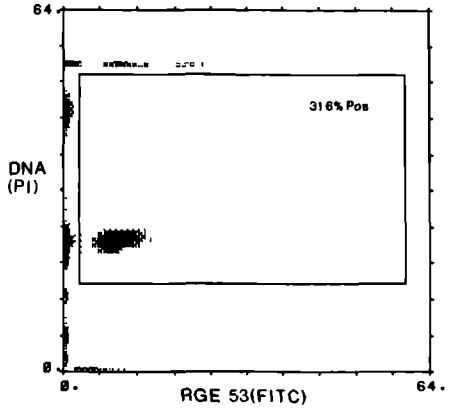
No	Age	Histological grade	Clinical stage	Used for SCM	Used for FCM
1	75	1	IA	yes	yes
2	50	1	IA	yes	yes
3	64	1	IA	no	yes
4	55	1	IB	yes	no
5	78	1-2	IA	yes	yes
6	72	1-2	IA	yes	yes
7	61	1-2	IA	yes	yes
8	73	1-2	IA	no	yes
9	44	1-2	IB	yes	yes
10	69	1-2	III	yes	yes
11	60	2	IA	yes	yes
12	86	2	IB	yes	yes
13	71	2	IB	yes	yes
14	44	2	II	yes	no
15	59	2	III	no	yes
16	69	2-3	IB	yes	yes
17	74	a	II	yes	yes
18	61	a	IV	yes	yes

SCM = scanning cytometry
FCM = flow cytometry
a = adenosquamous carcinoma

As a last step PI staining was performed.

Prelimary experiments showed that the RNAse treatment, necessary for PI DNA-staining, influenced later scanning nuclear protein measurements. Therefore the samples were either stained including the RNAse treatment for DNA flowcytometry, or without RNAse treatment for cell sorting and later scanning cytometry. In all cases chicken red blood cells were present in suspension as an internal standard for DNA determination. Cell analysis and sorting were performed using a cytofluorograph system 50H (Ortho Instruments, Westwood, MA). PI and FITC fluorescence was measured simultaneously and a two dimensional scatterplot was created (see Figure 1, top).A window was placed interactively in this plot to discriminate the RGE 53 positive cells (columnar epithelial cells) from the negative ones. For flow cytometry a DNA histogram was obtained of the cells within this window and plotted together with the chicken red blood cell internal standard, that was negative for RGE 53 (Figure 1, bottom).

Before the sorting procedure was started, the correctness of the window was checked by sorting 1,000 cells onto a slide. This was examined for PI and FITC fluorescence by fluorescence microscopy. Roughly estimated in all cases more than 90-95% of the cells were PI and FITC positive. Thereafter 10,000 of these cells were sorted into 21 µl 0,25% formaldehyde solution on xylene cleaned glass slides. After air drying these preparations were fixed in a methanol: 37% formaldehyde: acetic acid mixture (85:10:5, v/v/v) for one hour and stained for DNA and protein using Feulgen-Pararosanilin(SO_2) Light Green (9). A slide with chicken red blood cell nuclei was stained together with the clinical material as a control for possible staining variations. Feulgen-DNA and nuclear Light Green protein content were measured at random on two slides in hundred nuclei as described elsewhere (9), using an Axiomat scanning microscope (Zeiss, Oberkochen, W-Germany). Nuclear protein was defined as the protein measured within the contour of the nuclear area. This contour was determined in the nuclear image obtained from 565 nm wavelength measurements, the optimal wavelength for Feulgen-Pararosanilin(SO_2) determination. To detect eventual deviations from proportionality between nuclear protein and DNA, nuclear protein was divided by the DNA content for each cell. The mean and the coefficient of variation



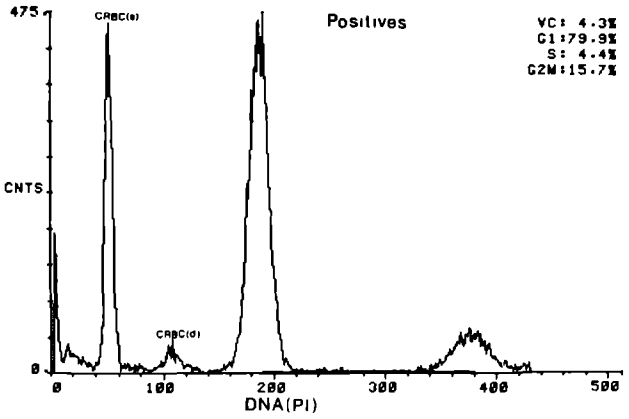


Figure 1. Top: Two parameter FCM analysis of cells from normal secretory endometrium stained with PI for DNA (ordinate) and with RGE 53, for the indirect immunofluorescence detection of cytokeratin 18 (abcissa). Limits of PI and RGE 53 positive cells are indicated in the figure. Chicken red blood cells have been used as an internal standard. These cells are negative for RGE 53 and are found on the ordinate before the G0/G1 fraction of the RGE 53 negative endometrial cells.

Bottom: PI-DNA histogram of the cells within the window from the top figure plotted together with the chicken red blood cell internal standard. CRBC(s) = chicken red blood cells, singlets; CRBC(d) = chicken red blood cells, doublets.

of the nuclear protein/DNA content of the hundred cells were calculated.

A DNA histogram was plotted comparable to that shown in a previous paper (11). In this histogram the position of the GO/G1 peak and the percentage of cells in the proliferative (S and G_2M) fraction were determined interactively, as described elsewhere (11).

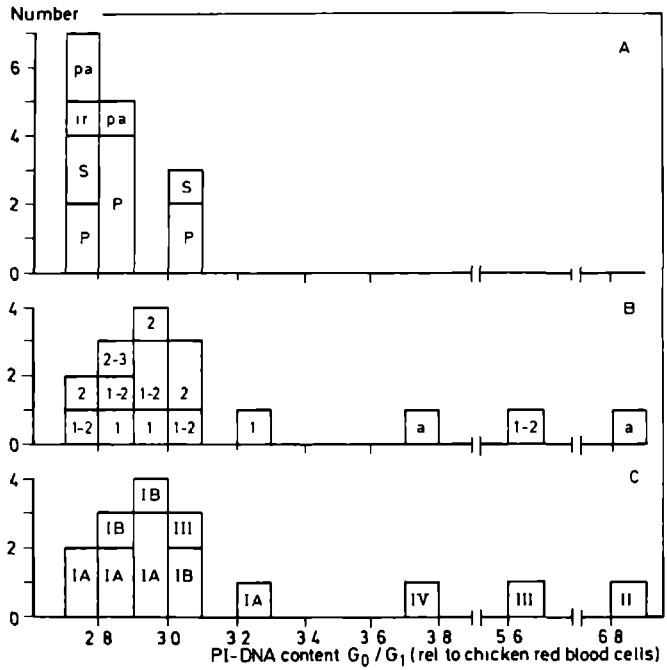


Figure 2. Histograms of the PI-DNA content of the GO/G1 fraction of columnar epithelial cells in normal (A) and malignant endometrium, classified according to histological grade (B) and clinical stage (C). In A "p" indicates proliferative, "s" secretory, "ir" irregular and "pa" postmenopausal atrophic. In B the grade and in C the stage are indicated in the bars. In B "a" indicates adenosquamous carcinoma.

Figure 2 shows histograms of the DNA-content of the GO/G1 fraction of the columnar cells in normal endometrium and endometrial adenocarcinomas, as determined by flow cytometry. The carcinomas are classified according to histological grade (figure 2B) and clinical stage (Figure 2C). All samples were stained and measured the same day, under identical circumstances. The PI-DNA content was devided by that of the chicken red blood cell internal standard. For human lymphocytes a ratio of 2.49 was found. Measurements of parallelly prepared and processed aliquots of the same sample showed a variation of less than 0.02 in this ratio. As can be seen an overlap is noted between the histograms, whereas the data of the carcinomas show a

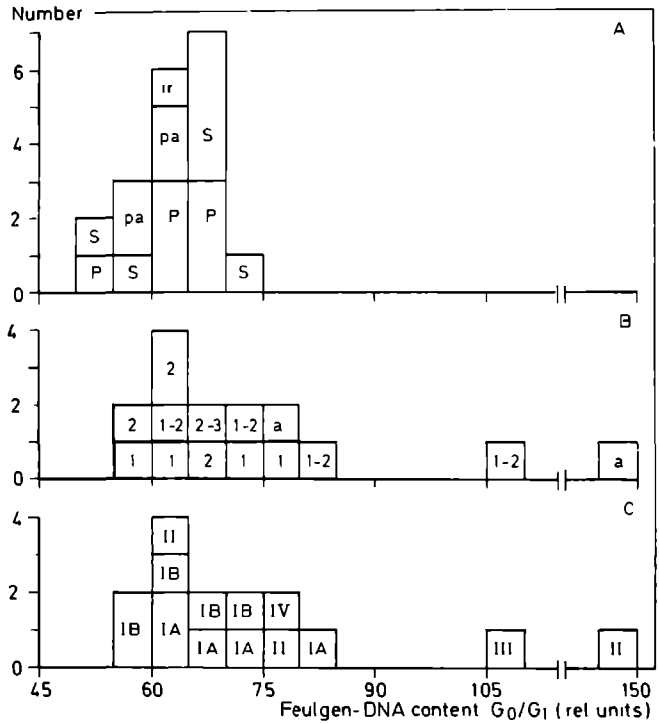


Figure 3. Histograms of the Feulgen-DNA content of the GO/G1 fraction of the columnar epithelial cells in normal (A) and malignant endometrium (B,C). For further details see figure 2.

shift towards higher values. Four carcinomas show a considerably increased DNA content, two of these are adenosquamous carcinomas. When clinical stage is considered three of the four with stage II and higher have a considerably increased DNA content.

Figure 3 shows histograms of the Feulgen-DNA content of sorted columnar cells from normal and malignant endometrium, as determined by scanning cytometry. The same tendency is found here as in case of the flow cytometrically determined DNA content. Normal and malignant endometrium show a considerable overlap with that of malignant endometrium shifted towards higher values.

Again the values for the adenosquamous carcinomas are found to be higher than that of normal endometrium and the same holds for 3 of the 4 carcinomas with clinical stage II and higher.

In figure 4, the percentages of cells in the proliferative fraction are shown for normal and malignant endometrial cells, determined from scanning cytometric DNA histograms.

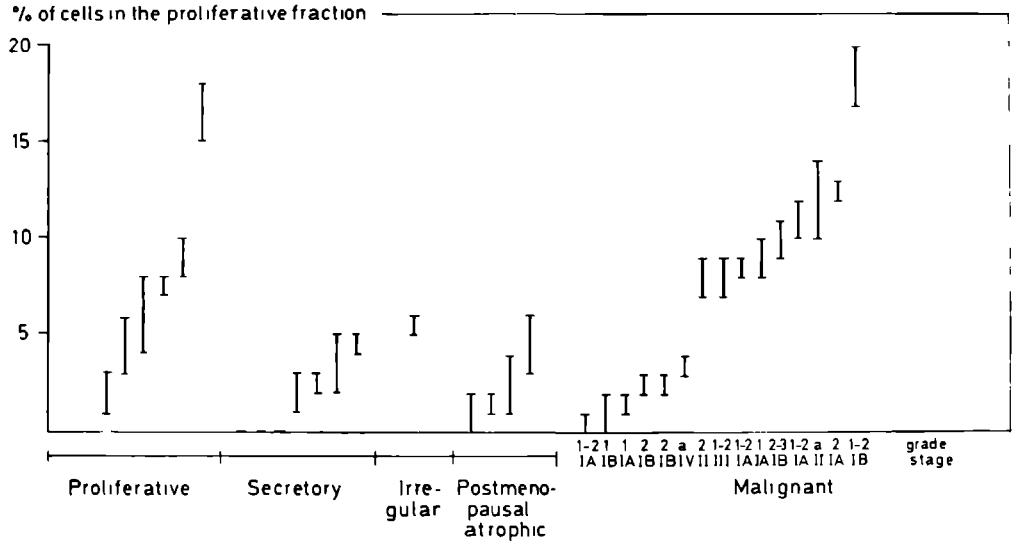


Figure 4. Percentage of columnar epithelial cells in the proliferative fraction of proliferative, secretory, irregular, postmenopausal atrophic and malignant endometrium. The minimal and maximal value per case are indicated. For malignant endometrium the number for grade and stage are indicated on the abcissa; "a" indicates adenosquamous carcinoma.

The minimum and maximum percentage are indicated per case because it is virtually impossible to indicate in the DNA histogram the border between the G0/G1 fraction and the S-phase. In normal proliferative endometrium values are found between 1 and 18%, whereas for normal secretory, irregular and postmenopausal atrophic endometrium values between 0 and 6% are found. For adenocarcinomas values between 0 and 20% are found. There was no correlation between the histological grade and the number of cells in the proliferative fraction. When the clinical stage was taken into account, 3 of the 4 carcinomas of stage II and higher were found in the "intermediate region".

Figure 5 shows histograms of the nuclear protein/DNA ratios as determined from scanning cytometric DNA-nuclear protein measurements. As can be seen there is a complete overlap between normal and malignant endometrium. For malignant endometrium there is also no clear correlation with the histological grade or clinical stage, with the exception of the two adenosquamous carcinomas. They are found towards higher values.

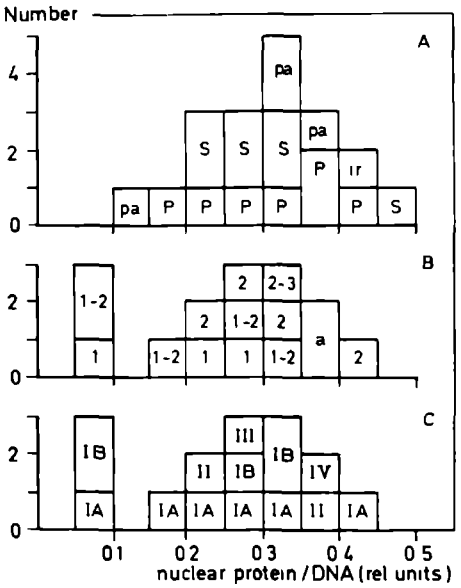


Figure 5. Nuclear Light Green protein/Feulgen-DNA histograms of columnar epithelial cells in normal (A) and malignant endometrium (B,C). For further details see figure 2.

Until now a comparison was made between normal and malignant endometrium based on a single parameter. Figure 6 shows a comparison in which two parameters were used, Feulgen-DNA content of the GO/G1 fraction and the coefficient of variation of the nuclear protein/DNA ratio. Using logistic discriminant analysis (1) a straight line could be constructed that discriminated normal endometrium from malignant. Only two normal endometria (both postmenopausal atrophic) are found in the "malignant region"

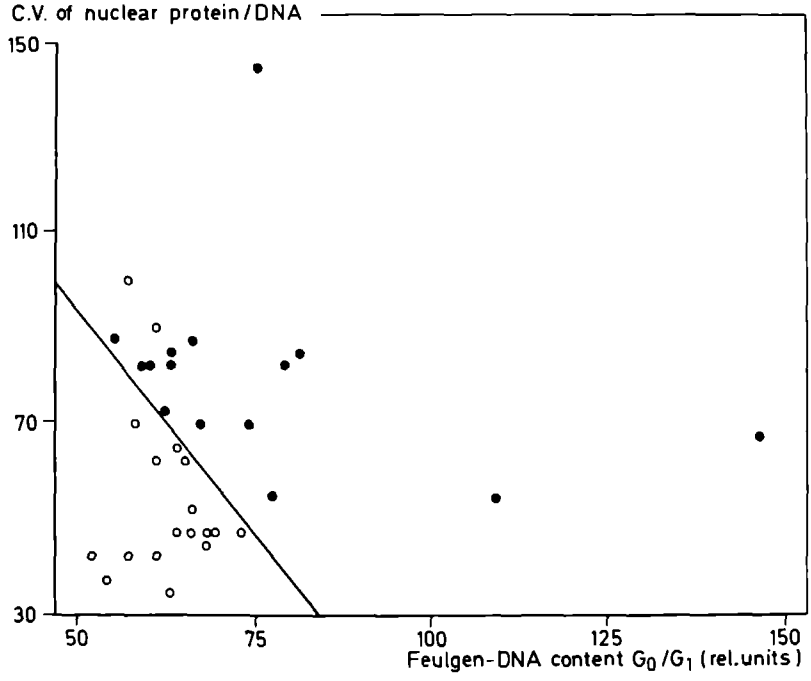


Figure 6. Two dimensional plot of the Feulgen-DNA content of the GO/G1 fraction (abcissa) and the coefficient of variation (c.v.)of the nuclear protein/DNA ratio (ordinate) of normal endometrium (o) and adenocarcinomas (●). Data were used from the samples shown in the figures 3 and 5.

In this study DNA and nuclear protein measurements were performed in columnar epithelial cells of normal and malignant endometrium. For DNA measurement both PI flow cytometry and Feulgen scanning cytometry were performed. With both staining techniques the same results were obtained. A considerable part of the adenocarcinomas have a comparable DNA content as normal endometrium irrespective of the histological grade. Only the adenosquamous carcinomas have a more increased DNA content. When clinical staging is used the majority of the stage I tumors is found in the diploid (normal) region whereas the more advanced stages have an increased DNA content. The aforementioned results were also found in a previous study where isolated nuclei of unselected endometrial cells and adenocarcinoma cells were compared (11).

Results from DNA flow cytometry of other tumors (2,8) especially the lymphomas and leukemias (3,4), have shown that a relationship may exist between the number of cells in the proliferative fraction and the histological grade. Also Feichter et al. (5) suggested that such a correlation might exist for endometrial adenocarcinomas. Therefore the percentage of cells in the proliferative fraction has been calculated from the scanning cytometric derived DNA histograms. This number varies considerably for the different adenocarcinomas and was not related to histological grade or clinical stage. The clinically more advanced carcinomas do not show an increased proliferative activity compared to the other carcinomas. These results closely agree with those found in a previous study (11).

The nuclear protein content as determined in this study does not seem to differ between normal and malignant endometrium. Also within the malignant population no indication could be found suggesting a correlation with the histological grade or clinical stage. These results are somewhat different from those obtained in our previous study (11). Then also a complete overlap of the nuclear protein content of normal and malignant endometrium was found. However, within the normal population the values of postmenopausal patients with atrophic endometria were found to be lower than those of patients with a proliferative and secretory endometrium. Within the malignant population the higher values were found in the clinically

more advanced stages (II and higher). In this previous study the measurements were performed in isolated nuclei of unselected cells. In the present study, however, the nuclear protein content was determined within the nuclear area of intact columnar epithelial cells. Thus, although a purification of the cells of interest was obtained, the level of discrimination seems to be reduced. A possible explanation for this reduction may be the interference in the measurements of cytoplasmic protein over and beneath the nucleus. Furthermore different degrees of nuclear protein loss during the isolation procedure used in this study and in our previous work may be a cause, although the conditions were chosen to minimize this effect.

The purpose of this study was to investigate the change of DNA and nuclear protein during neoplastic development, but also a possible application of the results for diagnostic use. As already mentioned, no discrimination between normal and malignant endometrium could be obtained on the basis of a single parameter. The use of the combined DNA-protein staining in this study allowed the simultaneous determination of DNA and nuclear protein in the same preparation. The combination of these parameters also did not result in a significant improvement in discriminative power (results not shown). However when DNA content was combined with the coefficient of variation of the nuclear protein content, a clear discrimination could be obtained, with only two cases of false positive results. Therefore, the use of extra parameters derived from DNA and nuclear protein measurements (such as the coefficient of variation) can result in a better discrimination, especially when they are used in a multivariate analysis. At present studies are in progress to investigate the use of other DNA derived parameters, such as the DNA-distribution in the nucleus (chromatin texture) (7,13), for optimal sample classification.

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SUMMARY

The application of new quantitative methods in pathology, such as image analysis and flow cytometry, has initiated the development of novel standardized specimen preparation procedures. The ultimate goal is to process specimens so that optimal measurements of already established, but also newly developed morphological and histochemical parameters can be performed. These procedures can be subdivided into cell processing and cell staining. In this thesis a number of cell processing (chapter 2) and cell staining (chapter 3) procedures are evaluated, for both morphological and histochemical analysis. The application of these methods in the histochemical analysis of normal and malignant endometrium is described in chapter 4.

In section 2.1 a cervical smear preparation procedure is described for quantitative morphological analysis. Cervical cells collected in a preservative solution were disaggregated by syringing, giving about 50% single cells. It was impossible to increase the percentage of single cells by the addition of different (bio)chemicals to the suspension. After counting and centrifugation the cells were resuspended in a carbowax solution, spread onto a glass slide and air dried. Standardization was achieved by keeping all handling very simple and by automating procedures such as the disaggregation of cell aggregates and cell clumps, the addition of the desired volume of carbowax solution and the spreading of the cells on the slide. Using these procedures a high number (approximately 100 by a technician per day) of cervical cell specimens of a quality permitting high resolution image cytometric measurements could be obtained.

For the optimization of this specimen preparation procedure a faster, more simple and yet equally effective method to disaggregate the cells was developed. This new disaggregation system, called the rotor device, is

described in section 2.2. A comparison was made with the syringing device, described in section 2.1. With the two devices similar results were obtained.

Another step in the cell preparation procedure that has been optimized was the transfer of the cells from suspension onto a glass slide. At first (section 2.3) the possibility of collecting cells on a slide through a filter intermediate was explored. Cells were collected on a polycarbonate filter and transferred to a slide by means of simultaneous pressure and fixation. The influence on cell recovery of filtration rate, filter pore size and duration of pressure application was analyzed. A fully automated system, called the Cytopress, based on this principle, is described in section 2.4. Cell recovery, the extent of contamination with foreign elements and the distribution of the cells over the slide were studied. The applicability of the technique to a variety of cytological material, other than cervical cells, is demonstrated. Examples presented are fine needle aspirations, monolayer and cell suspension cultures, agar cultures and isolated endometrial cell nuclei.

In addition to a proper cell processing procedure an adequate staining method is also required, for the measurement of relevant cell parameters. Sections 3.1 and 3.2 deal with staining methods applicable to image analysis and section 3.3 describes a method for flow cytometry.

In section 3.1 a staining method, Thionine-Feulgen Congo red, developed for use with a cathode ray tube scanner of photonegatives of cervical smears (BioPEPR), is described. The stain is a combination of the Feulgen procedure, with Thionine(SO_2) as an equivalent for the Schiff reagent, and Congo red, which is used as a cytoplasmic counterstain. The stain results in cell preparations suitable both for microscopic diagnosis and measurements performed on photonegatives made at a single wavelength of 545 nm. A high level of cervical cell

classification accuracy is reached in specimens stained according to this procedure.

Two DNA-protein stains, Feulgen-Pararosanilin(SO_2) / Light Green and Feulgen-Thionin(SO_2) / Orange II were evaluated in section 3.2. At first the protein stains Light Green and Orange II, and the Feulgen procedure for DNA with Thionine(SO_2) as an equivalent for the Schiff reagent, were investigated concerning specificity and stoichiometry. Next the influence of the protein post staining on the Feulgen procedure and the reverse, were studied. The amount of dye binding using the Feulgen procedure was hardly influenced by a following protein staining. However protein dye binding was reduced to a certain extent and depended on the DNA-protein staining combination and staining conditions in the Feulgen procedure.

The staining of endometrial cells in suspension with propidium iodide for DNA and a monoclonal antibody against a cytokeratin intermediate filament protein specific for columnar epithelial cells (RGE-53) was evaluated (section 3.3). Using flow cytometry DNA could be measured selectively in columnar epithelial cells of normal endometrium and endometrial adenocarcinomas. Also pure fractions of columnar cells sorted onto glass slides could be obtained and used for image analysis measurements.

DNA and nuclear protein measurements in nuclei of normal human endometrium and endometrial adenocarcinomas are described in chapter 4. Measurements were made on isolated nuclei obtained from tissue homogenates (section 4.1) or in isolated columnar epithelial cells sorted from disaggregated endometrial tissue in suspension (chapter 4.2), using preparation methods as described in earlier chapters.

The DNA content of nuclei of adenocarcinomas was equal to the DNA content of normal endometrium (section 4.1), or was slightly higher. In a few cases a marked increase was noted. No correlation was found with the histological

grade. The tumors in clinical stage II and higher had a higher DNA content than normal endometrium. The percentage of carcinoma cells present in the proliferative fraction, as deduced from DNA histograms, was equal to or higher than that of normal endometrium. No correlation was found with the histological grade or clinical stage.

The nuclear protein content of malignant endometrium did not differ from that of normal endometrium. However, in postmenopausal women most values of the carcinomas exceeded that of normal endometrium. Within the tumor series no correlation was found between nuclear protein content and histological grade. Higher values were found with tumors in clinical stages II and higher.

Endometrial adenocarcinomas are composed of malignant dedifferentiated columnar epithelial cells lining the surface and the glands of the endometrium. Therefore DNA and nuclear protein were also measured in this cell type only (section 4.2). DNA measurements completely agreed with those found for isolated nuclei. Such agreement was not found, however, for the nuclear protein measurements. No differences were found between normal and malignant endometrium and, within the carcinoma series, also no correlation with histological grade nor with clinical stage.

So, when using only one parameter, a discrimination could not be obtained between normal endometrium and endometrial adenocarcinomas. However the combined use of two parameters, derived from the combined DNA-(nuclear) protein stain, resulted in a good separation. The use of extra parameters deduced from this combined stain in the classification of adenocarcinomas of the endometrium is therefore recommended.

It can be expected that differentiation among the different types of endometrium carcinomas, using DNA and nuclear protein measurements, can be of prognostic value. Therefore, in a prospective study, the clinical behaviour of the endometrium carcinomas has to be correlated with the results of these image analysis measurements.

De toepassing van nieuwe meetmethodieken binnen de pathologisch anatomische diagnostiek, zoals beeldanalyse en flowcytometrie, heeft geleid tot de ontwikkeling van nieuwe, gestandaardizeerde cel- en weefselpreparatie technieken. Doel hiervan is het verkrijgen van preparaten, waarin optimaal reeds bekende en ook nieuwe morfologische en histochemische parameters gemeten kunnen worden. Deze technieken kunnen worden onderscheiden in celbewerkings- en celkleuringsmethoden. In dit proefschrift worden een aantal celbewerkings- (hoofdstuk 2) en kleurmethodieken (hoofdstuk 3) geëvalueerd, voor hun betrouwbaarheid bij zowel de morfologische als de histochemische analyse van cel- en weefselpreparaten. In hoofdstuk 4 wordt tenslotte de toepassing van deze methodieken bij de kwantitatief histochemische analyse van normaal en maligne veranderd endometriumweefsel beschreven.

Paragraaf 2.1 beschrijft de ontwikkeling van een methodiek voor het vervaardigen van celpreparaten van de baarmoederhals (cervix) voor kwantitatief morfologische analyse. Een optimale hoeveelheid van 50% losse cellen werd verkregen door de cervixcellen, gesuspendeerd in een preserverende oplossing, een aantal malen in en uit te spuiten door injectienaalden, middels een slangenpompsysteem. Het toevoegen van verschillende (bio)chemicaliën aan de preserverende oplossing had geen verdere verhoging van het percentage losse cellen tot gevolg. Na celtelling en centrifugatie werden de cellen geresuspendeerd in een fixerende carbowax oplossing. Vervolgens werd een bepaalde hoeveelheid van de uiteindelijke suspensie uitgestreken en aan de lucht gedroogd. Dit resulteerde in een gestandaardiseerde celopbrengst en een eenvoudige manier van verder verwerking van de preparaatglaasjes. Standaardisatie was verder verkregen door het merendeel van de handelingen zo

eenvoudig mogelijk te houden en/of te automatiseren, zoals het losmaken van de cellen, het toevoegen van het juiste volume carbowax en het uitstrijken van de cellen. Dit leidde er toe dat een analist per dag een groot aantal (gemiddeld 100) voor beeldanalyse kwalitatief hoogwaardige cervixcelpreparaten kan vervaardigen.

Om bovenstaande preparatiemethodiek, die ook toepasbaar is voor ander cyto(patho)logisch materiaal, verder te optimaliseren is gezocht naar een snellere, meer eenvoudige, maar even effectieve manier om de cellen van elkaar los te maken. Dit wordt beschreven in paragraaf 2.2, waarin een zogenaamd rotorapparaat wordt vergeleken met het celdisaggregeerapparaat beschreven in paragraaf 2.1. Dit nieuwe instrument bleek even effectief te zijn en veel sneller.

Een ander onderdeel van de celpreparatie methodiek dat verder geoptimaliseerd werd, was het overbrengen van de cellen vanuit suspensie op objektglaasjes. Automatisering en dus verdere standaardisering was hierbij het doel. Eerst werd de mogelijkheid bestudeerd om cellen via een filter intermediair op glas te brengen (paragraaf 2.3). Hiertoe werden cellen in een eerste stap middels filtratie op een polycarbonaat filter gebracht en vervolgens door gelijktijdige druk en fixatie op glas. Hierbij werd de invloed van parameters zoals filtratiesnelheid, gaatjes-diameter van het filter en tijdsduur van het overdrukken, op de uiteindelijke celopbrengst onderzocht. Vervolgens wordt (paragraaf 2.4) een volautomatisch apparaat, de zogenaamde Cytopress, beschreven dat ontwikkeld is op basis van het zojuist beschreven principe. De celopbrengst werd wederom bestudeerd, evenals andere relevante parameters zoals de mate van contaminatie en de verdeling van de cellen over het preparaatglaasje. Naast de toepassing in de cervix-cytologie wordt het gebruik van de methodiek beschreven voor dunnenaald aspiraten, cellen uit celkweken, celkolonies uit soft-agar cultures en geïsoleerde kernen uit endometriumweefsel.

Naast een gestandaardiseerde celpreparatie is er voor betrouwbaar kwantitatief morfologisch en histochemisch onderzoek eveneens een kleuringsmethodiek vereist die het mogelijk maakt relevante parameters van de cellen te meten. Voor beeldanalyse metingen worden deze beschreven in de paragrafen 3.1 en 3.2 terwijl een kleuringsmethodiek voor de flowcytometrie wordt behandeld in paragraaf 3.3.

De Thionine-Feulgen Congo Rood kleuringsprocedure, die ontwikkeld werd voor het analyseren van foto's van cervixuitstrijkpreparaten middels een kathodestraalbuis scanner (BioPEPR) wordt beschreven in paragraaf 3.1. De kleuring is een kombinatie van de voor het DNA specifieke Feulgen procedure, met Thionine(SO_2) als equivalent van het Schiff's reagens, en Congo Rood als cytoplasmatische tegenkleuring. Deze kleuring is zowel geschikt voor het gebruik bij de lichtmikroskopische diagnose, als voor metingen verricht aan foto's gemaakt met monochromatisch licht van 545 nm. Een hoge mate van juiste cervixcel classificatie werd gevonden in preparaten die volgens deze procedure zijn gekleurd.

Een evaluatie van twee DNA-eiwit kleuringen, Feulgen-Pararosaniline(SO_2) / Light Green en Feulgen-Thionine(SO_2) / Orange II, wordt beschreven in paragraaf 3.2. Hierin werden eerst onderzocht de eiwitkleuringen Light Green en Orange II, en de Feulgen procedure voor DNA met Thionine(SO_2) als equivalent van Schiff's reagens, op hun specifieke en stoichiometrische karakter. Vervolgens werd bij de gekombineerde DNA-eiwit kleuringen bestudeerd in hoeverre de Feulgen procedure invloed op de eiwitkleuring had en omgekeerd. Het bleek hierbij dat de intensiteit van kleuring van de Feulgen procedure niet of nauwelijks beïnvloed werd door de eiwitkleuring, maar dat de eiwitkleuring in meer of mindere mate gereduceerd werd door de voorafgaande Feulgen procedure. Dit laatste bleek afhankelijk te zijn van de gekozen DNA-eiwit kleuringskombinatie en de aangelegde kondities in de Feulgen-procedure.

Een methode om cylinderepitheelcellen uit

endometriumcelsuspensies te kleuren voor DNA met propidium iodide en cytokeratine 18 met behulp van een monoclonaal antilichaam (RGE-53) staat beschreven in paragraaf 3.3. Voor RGE-53 detektie werd een tweede antilichaam gebruikt waaraan een fluorochroom gekoppeld was. Op deze wijze kon DNA gemeten worden in zuivere populataties cylinderepitheelcellen van zowel normaal als maligne veranderd endometriumweefsel. Ook de mogelijkheid om deze cellen op objectglaasjes te sorteren en te gebruiken voor verdere beeldanalyse studies wordt aangeduid.

De resultaten van DNA en kerneiwit-metingen in celkernen uit normaal endometrium en adenocarcinomen van het endometrium worden beschreven in hoofdstuk 4. Deze bepalingen werden gedaan in geïsoleerde kernen verkregen uit homogenaten (paragraaf 4.1) en in geïsoleerde cylindercellen (paragraaf 4.2). Hierbij werden preparatiemethodieken gebruikt, die in de voorgaande hoofdstukken werden beschreven.
Het DNA-gehalte van kernen uit adenocarcinomen toonde een grote mate van overlap met dat van kernen uit normaal endometrium, of was enigszins verhoogd (paragraaf 4.1). In enkele gevallen werd een duidelijk verhoogd DNA-gehalte aangetroffen. Geen correlatie kon worden gevonden met de histologische graad van de tumor. Bij vergelijking met het klinisch stadium bleek dat de tumoren van stadium II en hoger een verhoogd DNA-gehalte hadden. Het percentage carcinoomcellen in de proliferatieve fase, berekend uit het DNA-histogram, was vergelijkbaar met dat van normaal endometrium of het was hoger. Er was geen relatie aantoonbaar met de histologische graad of het klinische stadium van de tumoren.

Het kerneiwitgehalte in de cellen van normaal en maligne veranderd endometrium vertoonde een totale overlap met elkaar. Echter bij post-menopausale vrouwen, bij wie het endometriumcarcinoom in verhoogde mate voorkomt, bleek het kerneiwitgehalte van carcinomen hoger te liggen. Binnen de groep van carcinomen was geen correlatie met de

histologische graad gevonden. Hogere waarden werden echter gevonden bij tumoren in het klinisch stadium II en daarboven.

Aangezien het adenocarcinoom van het endometrium ontstaat uit de cylindercellen die het oppervlak en de klierbuizen van het endometrium bekleden, zijn ook metingen verricht in opgezuiverde fracties van alleen dit celtype (paragraaf 4.2). De resultaten van de DNA-metingen kwamen geheel overeen met die gevonden zijn in geïsoleerde kernen. De resultaten van de kerneiwitmetingen verschilden echter. Ook hier werd geen onderscheid tussen normaal en maligne endometrium gevonden, maar bleek eveneens geen correlatie aantoonbaar binnen de carcinoomgroep, met de histologische graad en het klinische stadium.
Onderscheid tussen normaal endometrium en adenocarcinomen kon dus op basis van één parameter-metingen niet verkregen worden. Kombinatie van twee van de DNA- en kerneiwitmetingen afgeleide parameters resulteerde echter wel in een hoge mate van scheiding. Het gebruik van meer parameters, afgeleid van deze kombinatie kleuring, bij de klassificatie van adenocarcinomen van het endometrium wordt dan ook voorgestaan.

Verwacht mag worden dat differentiatie tussen de verschillende typen endometriumcarcinomen met behulp van DNA en kerneiwitmetingen prognostische betekenis heeft. In een prospectieve studie dient daarom het klinisch gedrag van de onderzochte endometriumcarcinomen te worden vergeleken met de resultaten van deze beeldanalyse metingen.

CURRICULUM VITAE

Peter Oud werd op 31 oktober 1951 te Enkhuizen geboren. Zijn HBS-B opleiding volgde hij aan de Rijks HBS in Enkhuizen waar in 1969 het diploma werd behaald. In 1969 begon hij aan zijn scheikunde studie aan de Universiteit van Amsterdam waar in 1972 het kandidaatsexamen S2 werd afgelegd. Het doktoraal examen Scheikunde met als hoofdvak Biochemie (Prof. dr. J.M. Tager) en bijvak Histochemie (Prof. dr. J. James) werd afgelegd in 1976.

Vanaf december 1976 tot december 1981 is hij als wetenschappelijk medewerker verbonden geweest aan het laboratorium voor Hoge Energie Fysica (Prof. dr. R.T. van de Walle) van de Wis- en Natuurkunde faculteit en het Instituut voor Pathologische Anatomie (Prof. dr. G.P. Vooijs) van de Geneeskunde faculteit van de Katholieke Universiteit van Nijmegen. Hij was daar werkzaam op een onderzoeksprojekt dat financieel ondersteund werd door het Koningin Wilhelmina Fonds. Vanaf januari 1982 tot heden is hij verbonden als wetenschappelijk medewerker aan het Instituut voor Pathologische Anatomie van de Katholieke Universiteit van Nijmegen, wederom (tot 1985) op een onderzoeksprojekt ondersteund door het Koningin Wilhelmina Fonds. Tijdens beide perioden werd het hier beschreven promotieonderzoek verricht.
Momenteel is hij werkzaam in een onderzoeksprojekt betreffende kwantitatief morfologisch onderzoek van het mamma-carcinoom, gefinancieerd door het Praeventiefonds.

In augustus 1976 is hij getrouwd met Tineke Jorritsma. Zij hebben samen drie kinderen: Matthijs, Ilse en Annemieke.

STELLINGEN

1. Optimaal kwantitatief pathologisch-anatomisch onderzoek kan uitsluitend worden uitgevoerd aan op gestandaardiseerde wijze geprepareerd cel- en weefselmateriaal, waarbij andere preparatiemethoden noodzakelijk zijn dan die in de routine pathologische anatomie gebruikelijk zijn.
(Dit proefschrift)

2. Bij de classificatie van maligne en pre-maligne afwijkingen middels kwantitatief pathologisch-anatomisch onderzoek kan het mede-gebruik van externe parameters, zoals de leeftijd van de patiënt(e) of het al dan niet gebruik van orale hormonale anticonceptie, tot een aanzienlijke verbetering leiden.
(Paragraaf 4.1, dit proefschrift. Zahniser, DJ: The development of a fully automatic system for the prescreening of cervical smears: BioPEPR. Proefschrift, Katholieke Universiteit Nijmegen, 1979)

3. Het is eenvoudiger in de kwantitatieve pathologische anatomie de kleurmethode af te stemmen op de meetmethode, dan omgekeerd.
(Hoofdstuk 3, dit proefschrift)

4. Het beeldanalytisch onderzoek zal uiteindelijk een veel belangrijker functie vervullen in de kwantitatieve pathologisch-anatomische diagnostiek dan het flow cytometrisch onderzoek.

5. Voor de differentiële diagnostiek van longtumoren is zowel lichtmicroscopisch als immunohistochemisch onderzoek vereist.

6. Het vervolgonderzoek bij vrouwen bij wie cytologisch een cervixafwijking Pap IIIA (passend bij een geringe of matige dysplasie) is vastgesteld, dient in eerste instantie cytologisch te zijn.

7. De aanwezigheid van de mononucleaire diploïde cel als stamcel van de parenchymcellen in de lever, naast de binucleaire diploïde cel en cellen van hogere ploïdie, maakt dit orgaan tot een geschikt model voor de bestudering van veroudering. (Van Noorden, CJF et al., Exp. Cell Res. 161:551-557, 1985)

8. De indeling van tumoren naar embryonale herkomst dient op grond van de gegevens verkregen bij immunohistochemisch en elektronenmicroscopisch onderzoek, waarbij een aantal tumoren zowel epitheliale als mesenchymale eigenschappen vertonen, te worden herzien.

9. Hoewel het ontstaan van het plaveiselcarcinoom in de vagina bij "oudere DES-dochters" in de lijn der verwachting ligt, komt deze afwijking slechts zeer zelden voor.

10. Het gebruik van de axillaire lymfeklierstatus bij de bepaling van de prognose van patiënten met een mammacarcinoom is slechts dan betrouwbaar, indien het onderzoek van de lymfeklieren op het voorkomen van metastasen op een gestandaardiseerde wijze wordt uitgevoerd.

11. De analist(e) fungeert dikwijls als het geweten van de wetenschappelijk onderzoeker.

12. Het dagelijks gemeenschappelijk consumeren van zelfgebakken taart, cake, koekjes e.d. heeft, ondanks de negatieve gezondheidsaspecten, een duidelijk positieve sociale functie.

Nijmegen, 20 februari 1986
P.S. Oud